2007

ROLE OF CALCIUM AND NITRIC OXIDE SYNTHASE (NOS) IN BRAIN MITOCHONDRIAL DYSFUNCTION

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ABSTRACT OF DISSERTATION

Vidya Nag Nukala

The Graduate School

University of Kentucky

2007

ROLE OF CALCIUM AND NITRIC OXIDE SYNTHASE (NOS) IN BRAIN MITOCHONDRIAL DYSFUNCTION

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine at the University of Kentucky

By

Vidya Nag Nukala

Lexington, Kentucky

Director: Dr. Patrick Sullivan, Associate Professor of Anatomy and Neurobiology

Lexington, Kentucky

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ABSTRACT OF DISSERTATION

ROLE OF CALCIUM AND NITRIC OXIDE SYNTHASE (NOS) IN BRAIN MITOCHONDRIAL DYSFUNCTION

Mitochondria are essential for promoting cell survival and growth through aerobic metabolism and energy production. Mitochondrial function is typically analyzed using mitochondria freshly isolated from tissues and cells because they yield tightly coupled mitochondria, whereas those from frozen tissue can consist of broken mitochondria and membrane fragments. A method, utilizing a well-characterized cryoprotectant such as dimethyl sulfoxide (DMSO), is described. Such mitochondria show preserved structure and function that presents us with a possible strategy to considerably expand the time-frame and the range of biochemical, molecular and metabolic studies that can be performed without the constraints of mitochondrial longevity *ex vivo*.

Mitochondrial dysfunction is implicated in Alzheimer's disease (AD) mainly through oxidative stress and altered metabolism. Mitochondria are isolated from post-mortem brain samples from selective regions of AD and control patients and, utilizing the cryopreservation strategy, analyzed for respiration and oxidative damage. While we did not observe increases in free radicals, we did observe decreased respiration and increases in oxidative damage markers in AD patients, suggesting a role for oxidative stress in mitochondrial dysfunction.

While in the mitochondria, calcium (Ca^{2+}) increases free radical generation by processes not completely understood. A new isoform of nitric oxide synthase (mtNOS) has been isolated and localized to mitochondria; though its existence and physiological role is debated. Nitric oxide synthase (NOS), when activated by Ca^{2+}, produces nitric oxide (NO•) that can interact with ROS producing various reactive nitrogen species (RNS). These highly reactive radical species can damage DNA, proteins and lipids, ultimately resulting in cell death via apoptosis or necrosis.

The current research is aimed at understanding the role of Ca^{2+} and NOS in oxidative stress leading to mitochondrial dysfunction. We observed a significant reduction in mitochondrial respiration with increasing doses of calcium. We also observed NOS enzyme activity and detected NOS protein in the purified mitochondrial fraction. Lastly, we were also able to show that Ca^{2+} increased the levels of free radicals and changes in oxidative damage markers. These results suggest the presence of NOS in mitochondria that could play a role in Ca^{2+} induced mitochondrial dysfunction and potentially leading to cell death as relevant to aging and neurodegenerative diseases.

KEYWORDS: Brain Mitochondria, Cryopreservation, Alzheimer's disease (AD), Calcium, Nitric Oxide Synthase and Oxidative Stress

VIDYA NAG NUKALA

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To All In Pursuit Of Knowledge

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Science builds on previous insights, and progress is made in small increments, in trying to understand the unknown. Research in particular constantly strives to question the prevailing dogmas and verify the current knowledge while leaving room for paradigm-shifting breakthroughs. Such endeavors help us discover what we are and what we could be. The work in this dissertation is driven by a curiosity to look into the fundamental mechanisms of human biology and health.

It has been made possible by the expertise, passion and encouragement of my mentor and dissertation chair Dr. Patrick Sullivan and I am grateful to him for that. He patiently taught me the workings of mitochondrial biology and made me appreciate their importance in human physiology and pathology. I thank my co-chair Dr. James Geddes for his critical questions and guidance during my dissertation work. My special thanks go to Dr. Edward Hall for being a constant source of wisdom and support for my endeavors, professional and otherwise. I thank Dr. Richard T. Miller for his expertise on NOS biology and for letting me avail his laboratory resources. I also would like to thank the members of my dissertation committee- Dr. Stephen W. Scheff, Dr. Michael B. Reid and outside examiner Dr. Rodney Guttmann for their valuable critique, comments and commitment.

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Chapter One

Mitochondria, Calcium, Nitric Oxide Synthase, Oxidative Stress, Cell Death and Aging

Mitochondria act as a biological switch in determining a cell's fate, in that they provide the necessary energy for the cell and in cases of noxious stimuli cause cell death by necrosis and/or apoptosis. The physiological function of mitochondria is to produce adenosine triphosphate (ATP), energy currency of the cell, through Kreb's cycle and electron transport chain (ETC). This ATP is utilized for various biological reactions inside the cell. Mitochondria are also involved in calcium (Ca^{2+}) homeostasis. However, as a consequence of ATP production, mitochondria also produce reactive oxygen species (ROS) as a by-product, mainly in the form of superoxide. The mitochondria are also equipped by anti-oxidant molecules and enzymes, and repair mechanisms. However, during pathophysiological conditions such as aging, neurodegeneration and neurotrauma, the balance between ROS and anti-oxidants is tipped causing oxidative stress and Ca^{2+} dysregulation. This leads to mitochondrial dysfunction, triggering a signaling cascade resulting ultimately in death of the cell (Pedersen 1999; Duchen 2000; Kroemer and Reed 2000; Newmeyer and Ferguson-Miller 2003; Smaili et al. 2003; Wallace 2005).

Mitochondria

Margulis, in one of the early theories of mitochondrial evolution in 1970, proposed that mitochondria originated from α-Proteobacteria that formed an endosymbiotic relationship with nucleocytoplasm of Archeon about 1.5 – 2.0 billion years ago (Gray et al. 1999; Lang et al. 1999; Gray et al. 2001; Searcy 2003; Dyall et al. 2004). However, studies of protists (unicellular eukaryotes) indicate that mitochondria originated at the same time as the eukaryotic nucleus

and not as a separate and subsequent event. In either case, genes necessary for amino acid biosynthesis, nucleoside biosynthesis, anaerobic glycolysis, cofactor biosynthesis, fatty acid and phospholipid metabolism, energy and intermediary metabolism, cell envelope synthesis, and cell division as well as most informational genes (genes directing replication, transcription, and translation) were typically transferred to host nuclear de-oxy ribo nucleic acid (DNA), thereby rendering a simple mitochondrial DNA (Gray et al. 1999; Wallace 2005).

Kolliker, in 1850, was among the first to describe characteristically arranged granules in the sarcoplasm of striated muscle, separated them from cell structure and noted they possess a membrane. They were termed “sarcosomes” by Retziius and “bioplasts” by Altman in 1890 and “mitochondria” (Greek; *mitos* meaning a thread and *chondros* meaning grain) by Benda in 1898. They were also known by various other terms such as blepharoblasts, chondriokonts, chondriomites, chondrioplasts, chondriosomes, chondriospheres, fila, interstitial bodies, mitogel, parabasal bodies, plasmasomes, spheroplasts, and vermicules, among others. Kingsbury and Warburg in 1912 studied mitochondria and demonstrated that they were involved with respiration, while Lewis and Lewis in 1914 found mitochondria to undergo changes in their size, shape and location. In the 1920s and 1930s, Szent-Gyorgyi (awarded the Nobel Prize in Physiology/Medicine in 1937) discovered the catalytic effect of the four-carbon dicarboxylic acids on respiration and Krebs (Nobel Prize in Physiology/Medicine in 1953) postulated the citric acid cycle as the primary cellular mechanism for the oxidation of carbohydrates, thus bringing mitochondria into the limelight as “powerhouses of the cell”.

Lohmann in 1931 discovered ATP, but its true significance in cellular energy requirements came about with the demonstration of Warburg in 1937–1938 of the formation of ATP coupled to enzymatic oxidation of glyceraldehyde phophate (and won the Nobel Prize in Physiology/Medicine in 1931) and the work of Meyerhof (Nobel Prize in Physiology/Medicine in 1922) on the formation of ATP from phosphopyruvate. During the same period, Kalckar demonstrated that oxidative phosphorylation was coupled to respiration. In 1940, Claude

investigated the structure, behavior, and chemical composition of mitochondria and microsomes isolated by differential centrifugation of liver homogenates (and won the Nobel Prize in Physiology and Medicine in 1974). During the period from 1943–1947, Leloir and Munoz, Lehninger and Kennedy, and Schneider and Potter conducted studies on oxidation of fatty acids in the liver, and established that mitochondria are indeed the sites of fatty acid oxidation, citric acid cycle and synthesis of ATP by oxidative phosphorylation (Nisoli et al. 2004).

Biogenesis of mitochondria has its roots in three theories: a) *de novo* synthesis of mitochondria from precursors present in the cytoplasm; b) formation from other membranous structures in the cell and c) growth and division of pre-existing mitochondria. Biogenesis of mitochondria can be initiated during mitosis or various external and internal stimuli such as inhibitors of oxidative phosphorylation, Ca^{2+} fluxes, thyroid hormone and low temperature. Mitochondria are also capable of undergoing frequent fusion and fission cycles, modulated by the mitofusins (Mfn) and dynamin-related proteins (Drp) respectively, thus displaying plasticity in their size, shape, number (or mass volume) and distribution. This ensures adequate availability of mitochondria to meet the energy requirements and limits the number of defective or damaged mitochondria. Mitochondrial biogenesis is brought about by transcriptional regulation. Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) and PGC-1β induce the expression of nuclear respiratory factors (NRF-1 and NRF-2), which in turn upregulate the expression of genes coding for both nuclear subunits of the respiratory chain and proteins involved in mitochondrial DNA transcription and replication. There is growing evidence of involvement of Ca^{2+} and nitric oxide acting as cellular sensors of energy expenditure and activating the mitochondrial biogenesis pathways (Nisoli et al. 2004; Nisoli et al. 2005).

The first high resolution images and structure of mitochondria were described by Sjostrand and Palade in 1952 and 1953 respectively. Mitochondria consist of an outer (OMM) and inner membranes (IMM) separated by the inter-membrane space (IMS). The inner membrane is impermeable to most molecules and consists of numerous invaginations called cristae, coined by Palade (who

later won the Nobel Prize in Physiology/Medicine in 1974), that house components of the ETC (Complexes I, II, III, IV) and ATP Synthase (Complex V). The inner membrane also encloses the matrix containing mitochondrial DNA (mtDNA), ribonucleic acid (RNA), ribosomes and several enzymes. In mammalian cells, each organelle contains several identical copies of mtDNA, whose structure and gene organization is highly conserved among mammals, each containing 37 genes that code for 2 ribosomal RNAs (rRNA), 22 transfer RNAs (tRNA) and 13 polypeptides (Taanman 1999; Szewczyk and Wojtczak 2002; Gibson 2005).

Another unique feature of mtDNA is its maternal inheritance in mammals. mtDNA is transferred through germline alone, when the sperm mitochondria are transferred to the oocytes during fertilization. All the mtDNA-coded polypeptides are subunits of the enzyme complexes of the ETC. The rest of the proteins are transcribed in the nucleus and imported into the mitochondria by an elaborate machinery of translocases located on the membranes of the mitochondria (Dyall et al. 2004; Wallace 2005). The outer membrane contains TOM (translocase of the outer membrane) complex for translocation and SAM (sorting and assembly machinery) complex for assembly of outer membrane proteins. The inner membrane contains TIM23 (translocase of the inner membrane) that mediates the import of mitochondrial matrix proteins with a typical N-terminal targeting sequence (positively charged on one side and hydrophobic on the other side and gets cleaved upon import), and the TIM22 complex mediates the import of inner membrane proteins (Koehler 2004).

One of the reasons for oxidative damage in mitochondria is increased susceptibility of mtDNA, owing to its proximity to ROS generation sites, absence of introns and lack of protective histones. This invariably leads to a very high mutation rate among mtDNA leading to a mixed intracellular population of mtDNAs, termed as heteroplasmy. Furthermore, in post-mitotic tissues, mtDNAs harboring deleterious mutations have been found to be preferentially, clonally amplified within cells. As the percentage of mutant mtDNAs increases, the mitochondrial energetic output declines, ROS production is elevated, and the

propensity for apoptosis increases. As cells are progressively lost through apoptosis, tissue function declines causing aging and/or movement disorders, dementias, cardio-vascular diseases and diabetes (Wallace 2005).

Mitochondria generate upto 95% of cellular ATP via oxidative phosphorylation carried out by the ETC. The ETC located on the IM of mitochondria comprises of Complexes I through IV and mobile electron carriers, coenzyme Q and cytochrome c. They are arranged in a series such that the electrons, donated by reducing equivalents from Kreb's cycle like nicotanamide adenine dinucleotide (NADH) or flavin adenine dinucleotides ($FADH_2$), flow down a voltage gradient, ultimately reducing oxygen to water at the level of Complex IV. In 1961, Mitchell proposed a mechanism for the coupling of electron transfer to ATP synthesis, based on an indirect interaction between oxidizing and phosphorylating enzymes. He suggested that the flow of electrons through the enzymes of the respiratory chain causes positively charged hydrogen ions, or protons, from the mitochondrial matrix being pumped by Complexes I, III and IV to the IMS of mitochondria. As a result, an electrochemical proton gradient is created across the membrane. The gradient consists of two components: a difference in hydrogen ion concentration (pH gradient) and a difference in membrane potential ($\Delta\psi$) of around 150mV; forming the 'protonmotive force'. The synthesis of ATP is driven by a reverse flow of protons down the gradient through ATP synthase (Complex V) resulting in generation of ATP from ADP (adenosine diphosphate) and inorganic phosphate (Pi). Mitchell's proposal has been called the 'chemiosmotic theory', which won him a Nobel Prize in Chemistry in 1978, giving rise to the field of 'bioenergetics' (Pedersen 1999; Nicholls and Ward 2000; Nicholls and Ferguson 2002; Newmeyer and Ferguson-Miller 2003; Wallace 2005).

Each of the components of the ETC undergoes a cycle of oxidation and reduction reactions resulting in energy changes. NADH donates electrons to NADH-ubiquinone oxidoreductase (Complex I), which transfers them to coenzyme Q (ubiquinone), accompanied by pumping of protons from matrix into the IMS. Complex I can be inhibited by rotenone and piericidin A. On the other

hand, $FADH_2$ bypasses complex I and donates electrons to Succinate-ubiquinone oxidoreductase (Complex II) instead, that can then transfer them to ubiquinone. Complex II can be inhibited by malonate and nitroproprionic acid (3-NP) (Nicholls and Ferguson 2002).

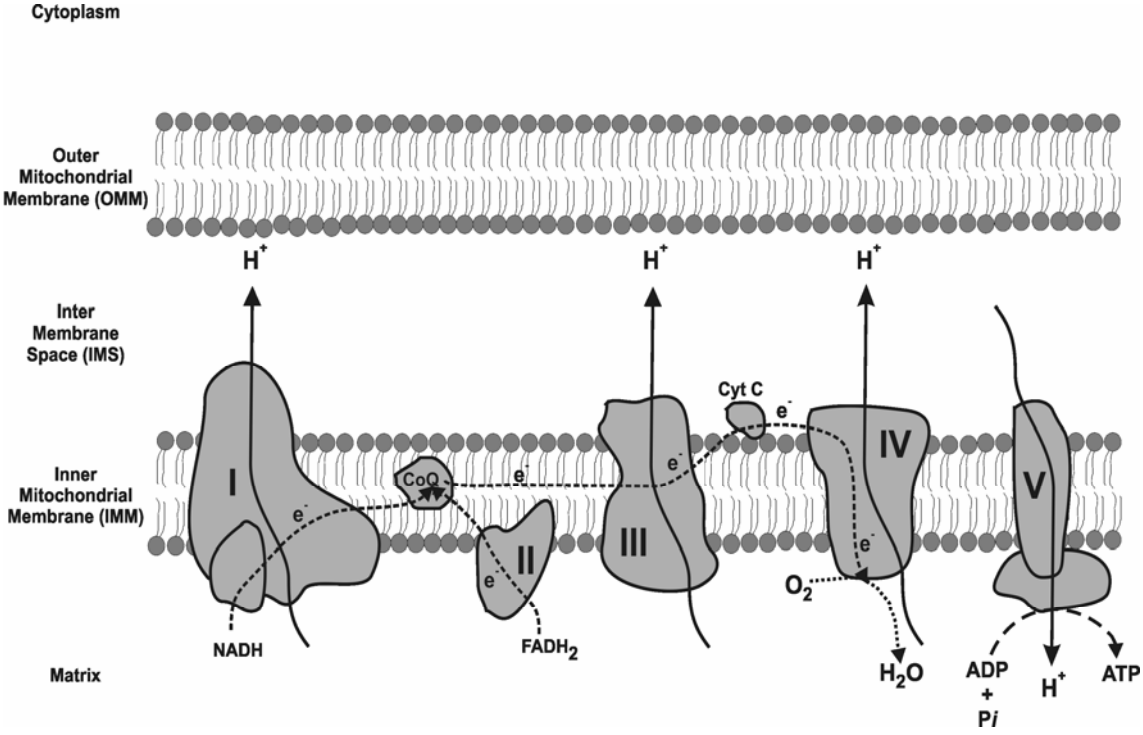
Coenzyme Q transfers electrons to cytochrome b component of Ubiquinone-cytochrome c oxidoreductase (Complex III or cytochrome c reductase or simply bc1 complex), which then reduces cytochrome c1 component. This leads to further pumping of protons into the IMS. Complex III can be inhibited by Antimycin A, myxothiazol and stigmatellin (Nicholls and Ferguson 2002).

The electrons are carried from cytochrome c1 of Complex III to cytochrome c oxidase (Complex IV or cytochrome a,a_3) by Cytochrome c located in the IMS, causing pumping of more protons into the IMS. Only cytochrome c oxidase is capable of overcoming the large kinetic barrier of simultaneous addition of four electrons to a molecule of oxygen that is then reduced to water (H_2O). Cyanide can inhibit Complex IV (Nicholls and Ferguson 2002).

F_0-F_1-ATPase or ATP synthase (Complex V) synthesizes ATP from ADP and P_i. This is achieved through reversing the ATPase in favor of synthase by consuming the energy of the proton gradient when protons flow back into the matrix from the IMS. This pioneering work on ATP synthase earned Boyer and Walker the Nobel Prize in Chemistry in 1997.ATP can then be transported out of the mitochondria by the ADP/ATP antiporter, adenine nucleotide translocase (ANT). Thus, flow of electrons down the voltage gradient is coupled to the proton gradient and subsequent phosphorylation of ADP to ATP (Nicholls and Ferguson 2002).

Figure 1.1: Illustration of the mitochondrial Electron Transport Chain (ETC). Electrons from NADH or $FADH_2$ FADH are passed through the ETC in the following order – a) NADH-ubiquinone oxidoreductase (Complex I) or Succinate-ubiquinone oxidoreductase (Complex II) b) coenzyme Q (ubiquinone) c) Ubiquinone-cytochrome c oxidoreductase (Complex III or cytochrome c reductase) d) Cytochrome c e) Cytochrome c oxidase (Complex IV or cytochrome a,a_3) and donated to oxygen reducing it to water. Protons are pumped from mitochondrial matrix to the inter-membrane space, that flow back through F_0-F_1-ATPase or ATP synthase (Complex V) resulting in generation of ATP.

Figure 1.1: Illustration of the mitochondrial Electron Transport Chain (ETC).



Calcium

The story of calcium (Ca^{2+}) began around 1883 with the publication of a paper by Sydney Ringer, who observed that Ca^{2+} is needed for maintaining heart contraction in the saline medium, now known as Ringer's solution. It was not until the 1930's that anyone paid much attention to this molecule. However, in 1940s, Heilbrunn proposed that Ca^{2+} by diffusion was one of the activators for intracellular processes, especially muscle contraction, that was also observed by Kamada and Kinoshita. In 1942, Bailey discovered that the ATPase function of myosin was activated by Ca^{2+} but not by magnesium (Mg^{2+}) and concluded that liberation of Ca^{2+} in the vicinity of ATPase was responsbible for muscle contraction. Sandow (1950) proposed that Ca^{2+} released from cortical or membrane regions of the muscle fibers activated actomyosin ATPase, a process he named "excitation-contraction coupling". Miledi's (1973) studies reporting release of neurotransmitters from presynaptic terminals, Kanno's group (1973) reporting exocytosis in mast cells, Timourian's group (1972) showing formation of cleavage furrow in oocytes, and Rose and Lowenstein's (1975) observations on the distribution of Ca^{2+} in the cytoplasm raised its status from just a structural element to one of the most important molecules required for biological activity (Carafoli et al. 2001; Carafoli 2002).

Calcium homeostasis is critical for cell survival and hence Ca^{2+} levels are maintained in the cytoplasm at a resting level of about 100nM, and can rise upto 1μM upon activation, as opposed to a concentration of approximately 1.2mM extracellularly (Berridge et al. 2000). It exhibits a wide-ranging spatial and temporal dynamics resulting in an extensive, but highly regulated, Ca^{2+} signaling network (Carafoli et al. 2001). At any given time, intracellular Ca^{2+} levels are maintained by a balance of 'on' reactions enabling Ca^{2+} to enter the cell and 'off' reactions resulting in its extrusion from the intracellular space, and the variations of on/off reactions generate Ca^{2+} transients. The 'on' reactions are activated primarily upon flow of Ca^{2+}, down its concentration gradient, from extracellular space, upon membrane depolarization via voltage-operated channels (VOC that

include L-, P/Q-, N-, R- and T-type channels) or agonist binding (glutamate, ATP, acetylcholine) via receptor-operated channels (ROC; ex: N-methyl-D-aspartate (NMDA) receptors) or store-operated channels (SOC) on the plasma membrane (PM). This initiates a signaling cascade engaging G-protein linked receptors, receptor tyrosine kinases (TyrK) and Phospholipase C (PLC). In a calcium-induced manner, Ca^{2+} can be released from internal stores such as endoplasmic reticulum (ER) predominantly via Ca^{2+}-sensitive inositol 1,4,5-trisphosphate receptors ($InsP_3R$) or ryanodine receptors (RyR) (Michelangeli et al. 2005).

Intracellular Ca^{2+} can activate Ca^{2+}-dependent enzymes such as protein kinase C (PKC), protein kinase A (PKA) and protein phophatases 1 and 2a (PP1 and PP2a), and through their phosphorylation/dephosphorylation functions can in turn modulate $InsP_3R$ and RyR activity on the ER. At the same time, most of the intracellular Ca^{2+} is sequestered and buffered by calbindin and calretinin in the cytosol, thereby fine-tuning the amplitude and duration of Ca^{2+} transients. Calcium can be buffered by the low affinity and high capacity calnexin and calreticulin inside the ER lumen, where it is required for chaperone-mediated protein folding. The Ca^{2+}-binding ratio (K_s) is defined as the ratio of amount of bound Ca^{2+} compared to Ca^{2+} that is free in the cytosol and varies greatly among different cell types. Calcium can also bind to sensor proteins such as calmodulin (CaM) and troponin C (TnC) or effector proteins like synaptotagmin, annexins, adenylyl cyclases, NOS, calpains among others and trigger a multitude of signaling cascades (Berridge et al. 2000; Berridge et al. 2003; Nicholls 2005). Calcium can also induce transcription by activating various transcription factors such as nuclear factor-kappaB (NF-κB), nuclear factor of activated T cells (NF-AT), cyclic-AMP response element binding protein (CREB) through NF-kappaB inhibitory protein (IκB), calcineurin and Ca^{2+}/Calmodulin-dependent calmodulin kinase II (CaMKII) respectively.

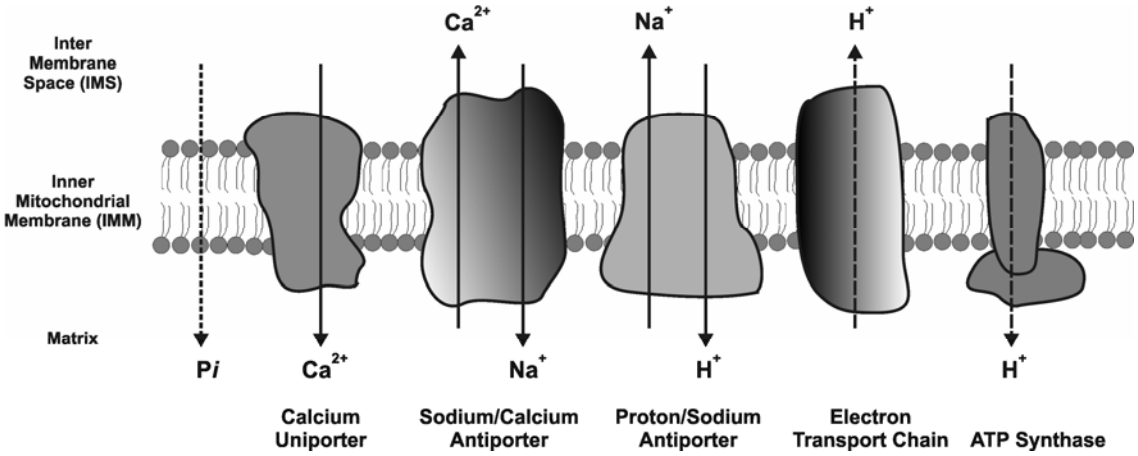
The ‘off’ reactions, on the other hand, include cytosolic calcium uptake by sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCA), secretory-pathway Ca^{2+}-ATPases (SPCA) on golgi and mitochondrial calcium uniporter (MCU), while calcium extrusion is achieved by Na^{+}/ Ca^{2+} exchangers (NCX) on mitochondria

and plasma membrane (PM) and plasma membrane Ca^{2+}-ATPases (PMCA) (Berridge et al. 2000; Berridge et al. 2003; Michelangeli et al. 2005; Saris and Carafoli 2005). The Ca^{2+} transients trigger off signaling cascades that can last from micro-seconds to a few hours and are responsible for synaptic exocytosis in learning/memory, muscle contraction, metabolism, transcription, fertilization, cell cycle, cell differentiation and development (Berridge et al. 2000; Berridge et al. 2003).

Mitochondria are one of the important organelles for intracellular calcium homeostasis and are equipped with a highly regulated Ca^{2+} transport system. They sequester Ca^{2+} rapidly during the development of Ca^{2+} signal and then release it back slowly during the recovery phase, thus shaping both the amplitude and the spatio-temporal patterns of Ca^{2+} signals. Slater and Cleland (1953) first observed that isolated mitochondria could take up large amounts of Ca^{2+} even at 0°C. Chance (1955) noticed that Ca^{2+} stimulated State 4 respiration in isolated mitochondria and concluded that Ca^{2+} was being 'consumed' during uncoupling phase. This was followed by the finding, in 1959 by Saris, that addition of Ca^{2+} to isolated mitochondria acidified the external medium, leading the author to conclude that proton (H^+) and Ca^{2+} gradients were produced due to uptake of Ca^{2+} by mitochondria. Eventually, in 1961-62, Vasington and Murphy measured directly the uptake of Ca^{2+} by isolated kidney mitochondria coupled to oxidative phosphorylation, and extended the observation by showing that the uptake required respiration and inorganic phosphate. De Luca and Engstrom (1961) had shown that the process of energized Ca^{2+} uptake was an alternative to ADP phosphorylation in the usage of respiratory chain energy. Lehninger's group (1963) pointed out that inorganic phosphate was accumulated together with Ca^{2+} and a carrier-mediated process was proposed for the uptake of Ca^{2+} by Lehninger and Carafoli in 1969. A second pathway separate from that catalyzed by the Ca^{2+} uniporter has been described recently, a "rapid" mitochondrial (RaM) Ca^{2+} uptake pathway by Gunter's group in 1998, which is transiently activated by high [Ca^{2+}].

Figure 1.2: Illustration of mitochondrial ion transport. Ion transport in mitochondria is strictly enforced by a set of tranporters and pumps. Calcium (Ca^{2+}) enters the mitochondria through a Ca^{2+} uniporter, at the expense of mitochondrial membrane potential ($\Delta\psi$). Calcium leaves the mitochondria through a sodium-linked (Na^{+}) antiporter, bringing Na^{+} into the mitochondria. Sodium, on the other hand, leaves mitochondria through the Na^{+}-H^{+} (sodium-proton) pump. Lastly, protons are pumped into the inter-membrane space (IMS) through Complexes I, III and IV and flow back into the matrix through Complex V or ATP-Synthase.

Figure 1.2: Illustration of mitochondrial ion transport.



The excess cytosolic calcium is sequestered into mitochondria via an electrogenic Ca^{2+} uniporter that is driven by $\Delta\psi$ and has a low sensitivity for Ca^{2+} (Nicholls and Chalmers 2004). This requires the mitochondria to be in close proximity to calcium-releasing channels of ER (Sorrentino and Rizzuto 2001; Hajnoczky et al. 2003) or PM. Work by Benton's group in 1970's showed an all important physiological function for Ca^{2+} is that it regulates the citric acid cycle by activating pyruvate dehydrogenase, NAD^{+}-dependent isocitrate dehydrogenase, α-ketoglutarate dehydrogenase complex (KGDHC) as well as ATP synthase, causing increases in respiration and ATP production (Gunter et al. 2004), in the 0.1 to 1μM concentration range. Calcium inside the matrix tends to form Ca^{2+}-phosphate compound that is readily dissociable, which keeps the free matrix Ca^{2+} at low levels (Nicholls and Chalmers 2004).

The Na^{+}-linked efflux pathway was first identified in heart mitochondria by Carafoli's group in 1974 and was subsequently shown to be the primary Ca^{2+} efflux mechanism of most mitochondria. In 1979, Carafoli proposed the 'mitochondrial Ca^{2+} cycle' - the continued operation of the uptake and release limbs of the transport process results in a futile cycle, in which respiratory energy is dissipated. This efflux occurs till the extra-mitochondrial concentration is around 1μM known as the 'set-point'.

Calcium is taken up by mitochondria if the concentration is above the set-point and released if below (Nicholls and Chalmers 2004; Nicholls 2005). In addition to Ca^{2+} efflux via specific transporters, the inner mitochondrial membrane may under certain conditions undergo permeability changes, observed by Beatrice's group in 1980, leading to the loss of membrane potential and to the leakage of small ions, induced by high Ca^{2+} concentrations. It has been suggested that they are mediated by a high conductance unselective channel, named permeability transition pore (PTP) and the proposal that mitochondria could protect the cytosol against the damage induced by excessive Ca^{2+}. Thus, mitochondria play a key role in Ca^{2+} homeostasis. However, calcium dysregulation has been shown to be involved in mitochondria-mediated cell

death (Nicholls and Ward 2000; Yu et al. 2001; Hajnoczky et al. 2003; Orrenius et al. 2003; Nicholls 2005).

Nitric oxide synthase (NOS) and Nitric oxide (NO)

The significance of nitric oxide synthase (NOS) and its product nitric oxide or nitrogen monoxide (NO•) is exemplified by awarding of the Nobel Prize in Physiology/Medicine to Furchgott, Ignarro and Murad in 1998 and recognizing Moncada as the most highly cited UK biomedical scientist in 1999 (Alderton et al. 2001). Furchgott, in 1980, made the observation that vascular smooth muscle relaxation was dependent on a diffusible substance released by the surrounding endothelium and called it the endothelium-dependent relaxation factor (EDRF) with a short half-life that can be destroyed by superoxide ($O_2^{\bullet -}$). Nitrovasodilators, such as glyceryl trinitrate and sodium nitroprusside, could cause vascular smooth muscle relaxation, by stimulation of guanylyl cyclase and increase in cyclic guanosine monophosphate (cGMP), shown by Murad, and could be inhibited by haemoglobin. Ignarro and Moncada independently confirmed, in 1987, that NO• was indeed the EDRF. Moncada's group also showed that NO synthesis in endothelial cells requires the amino acid L-Arginine. The first NOS protein was isolated and purified by Bredt and Snyder in 1990 (Vallance 2001; Stuart-Smith 2002; Clementi 2003).

Nitric oxide synthase (NOS) enzyme is one of the most highly conserved proteins across species and plays a critical role in the normal physiology of the body via its major product nitric oxide (NO•). Three distinct NOS isoforms have been identified namely – neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3). The human nNOS gene has been localized to chromosome 12 and its protein product is 161kDa, with multiple splice variants, found mainly in the cytosol of neuronal, skeletal and cardiac muscle cells, neutrophils, pancreatic islets. eNOS gene has been localized to chromosome 7 with a protein product of 133kDa and, owing to its myristate and palmitate groups at its N-terminus, is present bound to plasma membrane of

endothelial cells, smooth muscle and neurons. Both nNOS and eNOS are constitutively present and activated by binding of Ca^{2+} and calmodulin (CaM). On the other hand, iNOS gene is present on chromosome 17 coding for a protein product of 131kDa, and is predominantly found in the cytosol of macrophages, neurons, endothelial and other cell types. It is Ca^{2+}-independent for its activation since it is tightly bound by CaM and mainly controlled at the level of gene transcription in response to challenges to the immune system (Forstermann et al. 1995; Geller and Billiar 1998; Hobbs et al. 1999; Guix et al. 2005).

The X-ray crystal structures of NOS domains were made available in 1998 and 1999. The primary structure of NOS consists of an N-terminus oxygenase linked to a C-terminus reductase domain by Ca^{2+}/CaM binding site and is found typically as a homodimer. The oxygenase domain contains binding sites for L-Arginine, heme and (6R)-5,6,7,8-tetrahydro-L-biopterin (BH_4). Nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (Fadeel and Orrenius) and flavin mononucleotide (FMN) have their binding sites in the reductase domain at the C-terminus. In presence of Ca^{2+}/CaM bound to the NOS, electrons from NADPH are donated to the reductase domain of the enzyme and proceed via FAD and FMN redox carriers to the oxygenase domain. They interact with the haem iron and BH_4 at the active site of the oxygenase domain to catalyze the reaction of oxygen with L-arginine, generating L-citrulline and NO• as products (Forstermann et al. 1995; Bryk and Wolff 1999; Hobbs et al. 1999; Groves and Wang 2000; Alderton et al. 2001; Guix et al. 2005). NOS can be post-translationally regulated by various mechanisms such as dimerization, sub-cellular localization, phosphorylation, availability of substrates and cofactors, feedback inhibition and proteolytic degradation (Forstermann et al. 1995; Alderton et al. 2001).

Figure 1.3: Illustration of various Nitric Oxide Synthase (NOS) isoforms. Three well-known isoforms of Nitric Oxide Synthase (NOS) are depicted below. Each of the isoform contains a distinct oxygenase and reductase domains with various sites for binding of substrates and cofactors. In addition, neuronal NOS (nNOS or NOS-1) contains a PDZ domain and endothelial NOS (eNOS or NOS-2) contains myristic and palmitate groups near their N-terminus, both of which are absent in inducible NOS (iNOS or NOS-3).

Figure 1.3: Illustration of various Nitric Oxide Synthase (NOS) isoforms.

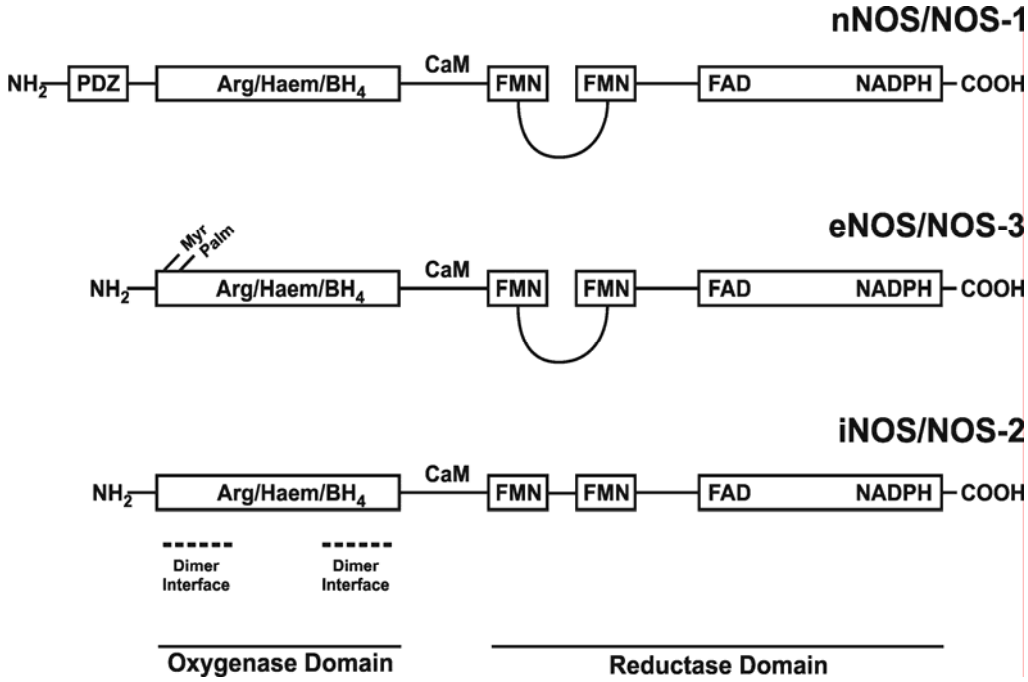
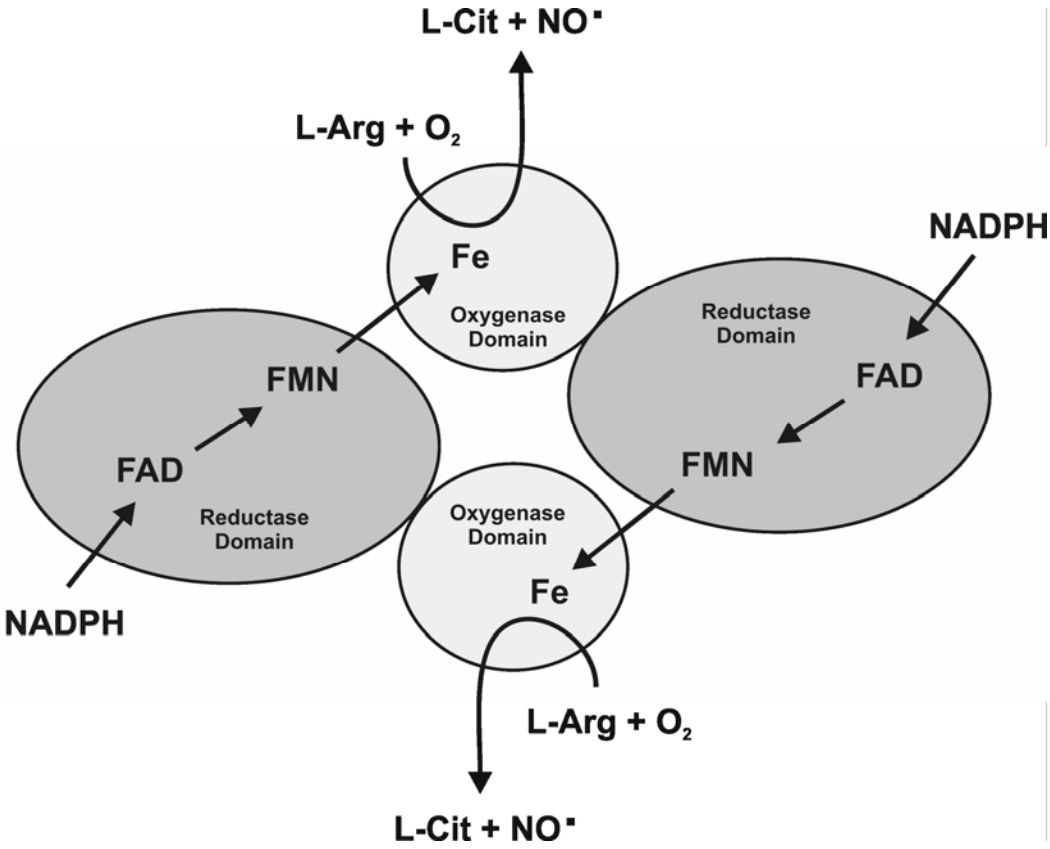


Figure 1.4: Illustration of Nitric Oxide Synthase (NOS) enzyme reaction. Electrons are donated by NADPH to FAD in the reductase domain of one monomer. They are passed onto FMN and then to haem iron (Fe) bound to the oxygenase domain of the other monomer in the homo-dimer required for activity. In presence of cofactors (Ca^{2+}/CaM, BH_4), the electrons are then donated to the NOS substrate L-Arginine (L-Arg) to react with O_2 forming L-Citrulline (L-Cit) and nitric oxide (NO•).

Figure 1.4: Illustration of Nitric Oxide Synthase (NOS) enzyme reaction.



In neuronal synapses, nNOS typically interacts, through its unique N-terminal PSD-95 discs large/ZO-1 homology (PDZ) domain, with the PDZ domain of post-synaptic density protein 95 (PSD-95) that in turn binds to NMDA receptors and regulates the entry of Ca^{2+} into the cell. Glutamate-induced Ca^{2+} entry into the cell results in a Ca^{2+}/CaM complex that then translocates to the PM and binds to nNOS, where the binding is enhanced by heat-shock protein 90 (Hsp90). Calcineurin dephosphorylates and activates nNOS, which then translocates to the cytosol where it produces NO•. Nitric oxide (NO•) activates guanylyl cyclase resulting in production of cyclic guanosine monophosphate (cGMP) that in turn activates various signaling pathways while PKA or PKC can inactivate nNOS by phosphorylation. Alternately, nNOS activity is proposed to be inhibited by protein inhibitor of NOS (PIN) which dissociates the dimerized form of NOS while others have proposed that PIN only affects translocation of nNOS (Alderton et al. 2001; Kone et al. 2003).

Endothelial NOS (eNOS) is myristoylated and palmitoylated and trafficked to caveolae (micro-domains of plasma membrane invaginations) of endothelial cells. It is inserted into the caveolae membranes and exists in an inactive state when bound to caveolin-1. Activation of endothelial acetylcholine receptors in turn activates phospholipase C (PLC) that catalyzes the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2). The IP_3-induced increase in intracellular Ca^{2+} activates calmodulin binding to eNOS, which then dissociates from caveolin and translocates to the cytoplasm. Heat-shock protein 90 (Hsp90) in this case is shown to act as an allosteric activator of eNOS, and together with CaM and caveolin form a dynamic ternary complex. Phosphorylation by protein kinase B (Akt) activates eNOS while phosphorylation by protein kinase A (PKA) inactivates the enzyme and is sequestered away from caveolae with diminished activity by eNOS-interacting protein (NOSIP) and eNOS traffic inducer protein (NOSTRIN). Finally, a fall in Ca^{2+} levels dissociates CaM from eNOS, and the inactive form relocates to the membrane caveolae. Both nNOS and eNOS activities also seem

to be regulated by certain G-protein coupled receptors such as Bradykinin B_2 receptor by binding and inhibiting them (Alderton et al. 2001; Kone et al. 2003).

Upon cytokine interferon-γ (IFN-γ) binding and activation of IFN-γ receptors, the signal is passed onto the Janus kinase (JAK) family and signal transducers and activators of transcription (STAT) proteins. Receptor occupation and dimerization induces the phosphorylation of associated STATs. Activated STATs dimerize and translocate to the nucleus where they increase expression of the transcription factor, IFN-γ regulatory factor 1 (IRF-1), that in turn binds to specific DNA elements in the iNOS gene promoter region to increase iNOS gene expression. Its activity is found to be increased by Rac1 and Rac2 of Rho-like GTPases family. On the other hand, iNOS protein is regulated by 3 inhibitory proteins namely a) NOS-associated protein 110 (NAP-110) b) kalirin and c) caveolin. While the former two proteins inhibit iNOS activity by preventing its dimerization, caveolin binds to iNOS and tags it for proteolysis (Kone et al. 2003). Lastly, nNOS and iNOS are targeted for degradation mostly by calpains or the ubiquitin-proteasome pathway, the choice of pathway and mechanisms needing further elucidation (Osawa et al. 2003).

Nitric oxide (NO•) was declared “The Molecule of the Year” by Science magazine in 1992. Nitric oxide acts a local hormone owing to its solubility in lipid and water, being a diatomic free radical and having short half-life of a few seconds in biological fluids. This allows NO• to diffuse across membranes (with a diffusion coefficient 1.4 times that of oxygen at 37°C) and act as a messenger within a radius of hundred microns. *In vivo*, the concentration of NO• can range from 5nM to 4μM and is buffered and transported by oxyhaemoglobin in red blood cells and NO• mediates its effects by acting on compounds containing transition metals, such as iron-sulphur (Fe-S) clusters. Most of these are transcription factors, while it also reacts with four haem-containing enzymes namely heme-oxygenase 1 (HO-1), cytochrome c oxidase (COX-IV), catalase and soluble guanylyl cyclase (sGC) (Hanafy et al. 2001; Ridnour et al. 2004).

Aconitase and hypoxia-inducible factor 1 (HIF-1) are among the NOS-regulated transcription factors. Aconitase serves two roles in the cell; it can

function as a soluble enzyme in the Krebs cycle and as an iron homeostatic sensor. When iron is plentiful, a tetranuclear iron-sulfur center (4Fe-4S) aconitase is active as an enzyme in the Krebs cycle, which functions as an isomerase regulating the equilibrium between citrate and isocitrate. However, when the serum iron is depleted, there is no longer sufficient iron to form the tetranuclear iron-sulfur center. Aconitase in the absence of its 4Fe-4S center is called an iron-regulatory protein (IRP). To maintain iron homeostasis, the IRP binds to iron-responsive elements (IREs) and stabilize transferrin receptor mRNA, while they inhibit translation of ferritin mRNA. Ferritin is the major iron storage protein found in bone marrow. Increasing transferrin receptor and decreasing ferritin will increase serum iron. Nitric oxide synthase (NOS) also mediates regulation of the angiogenic transcription factor HIF-1. HIF-1 is a heterodimeric complex comprising of HIF-1a and ARNT (aryl-hydrocarbon receptor nuclear translocator)]. Under hypoxic conditions or NO• donation, HIF-1 binds to its response element and stimulates transcription of vascular endothelial growth factor (VEGF), insulin-like growth factor II (IGF-II) and erythropoietin (Hanafy et al. 2001; Droge 2002; Ridnour et al. 2004).

Heme-oxygenase 1 (HO-1) catalyzes the degradation of the heme moiety into biliverdin, carbon monoxide, and iron. HO-1 mRNA is destabilized by NO•, and since NOS is a heme requiring enzyme, a negative feedback loop is formed. Therefore, the cell has a system to keep NO• levels from becoming toxic. Cytochrome c oxidase (COX) is the final component of the electron transport chain where oxygen is reduced to water. Since NO• reacts on a similar time scale as oxygen diffusion, it acts as a competitive inhibitor to oxygen binding and thus reversibly inhibits COX. Catalase, a ferric-containing heme moiety, is involved in the reduction of hydrogen peroxide to oxygen and water. NO• binding to the ferric ion results in inhibition of catalase activity (Hanafy et al. 2001; Droge 2002; Ridnour et al. 2004).

Most of NO• effects are mediated by soluble guanylyl cyclase (sGC). Soluble guanylyl cyclase is a heterodimer composed of α- and β subunits. Upon binding of NO•, sGC catalyzes the conversion of 1 mole of guanosine 5’-

triphosphate to 1 mole of 3',5'-cyclic guanosine monophosphate and 1 mole of pyrophosphate. Cyclic GMP mediates its effects through 4 targets: cGMP-dependent protein kinase (PKG), cyclic nucleotide-gated channels (CNG), cAMP-dependent protein kinase and phosphodiesterase (Hanafy et al. 2001; Droge 2002; Ridnour et al. 2004).

Thus, NOS-derived NO•, at physiological levels, is credited with a role in angiogenesis, peristalsis, micturition, muscle contraction, wound healing, cardiac development, reproduction, learning/memory and apoptosis. Neuronal NOS derived NO• in the central nervous system (CNS) is shown to be involved in neurotransmission and neuronal plasticity; while in the peripheral nervous system (PNS), NO• from nitrergic nerves is involved in pain perception and mediates smooth muscle relaxation affecting gastro-intestinal motility and penile erection. In the skeletal muscle, nNOS derived NO• is suggested to be involved in regulation of myotube development, innervation and contractility (Bryk and Wolff 1999; Guix et al. 2005). nNOS knockout mice exhibit aggressiveness, pyloric sphincter stenosis, resistance to ischemia and stroke (Mashimo and Goyal 1999; Vallance 2001; Mungrue et al. 2003).

Endothelial NOS derived NO• is shown to maintain blood pressure and flow in the vasculature by smooth muscle relaxation through its action on soluble guanylyl cyclase, as well as inhibiting platelet aggregation. Nitric oxide from both eNOS and nNOS is suspected to be acting as a retrograde messenger involved in long-term potentiation (LTP) in brain hippocampus. Majority of eNOS knockout mice die shortly after birth due to cardiovascular abnormalities. Lack of NO• causes hypertension and enhances the formation of atherosclerotic lesions in the vessel walls of eNOS knockout mice, while overproduction of NO• itself can cause profound vascular relaxation resulting in acute drop in blood pressure (Bryk and Wolff 1999; Mashimo and Goyal 1999; Vallance 2001; Mungrue et al. 2003; Guix et al. 2005).

Inducible NOS (iNOS), on the other hand is transciptionally upregulated in macrophages, hepatocytes, astrocytes and other cell types by various agents such as lipopolysaccharide (LPS), cytokines and other microbial products.

Should such a situation arise, iNOS produces large amounts of NO• and its derivatives that are toxic to tumors and invading pathogens as well as promotes would healing and angiogenesis (Bryk and Wolff 1999; Guix et al. 2005). However, iNOS knockout mice show increased susceptibility to bacterial and viral pathogens, tumors and resistance to sepsis-induced hypotension (Mashimo and Goyal 1999; Vallance 2001; Mungrue et al. 2003).

Taken together, there is redundancy in the source and actions of NO• in normal physiology which also accounts for compensatory mechanisms in the event of deficiencies in one or more the NOS isoforms. Finally, NO• is also shown to result in formation of reactive nitrogen species (RNS) such as nitroxyl (NO^-) radicals or peroxynitrite ($ONOO^-$), causing nitrosylation/nitration of key proteins thereby contributing to oxidative/nitrosative/nitrative stress and subsequent cell death (Nicotera and Melino 2004) implicated in several pathologies (Groves 1999; Hobbs et al. 1999; Szabo 2003; Guix et al. 2005).

ROS/RNS and Oxidative Stress

The properties of what constituted air for life has intrigued humans since recorded history as noted in a Sumerian creation myth stating: “for the sake of the good things in their pure sheep-folds, Man was given breath.” Da Vinci, in the late 15th century, observed that air incapable of combustion was also incapable of supporting life, which he called the ‘vivifying spirit’. Mayow, in 1673, stated that “an aerial something essential to life, whatever it may be, passes in to the masses of blood”. In the 18th century, Priestley, Lavoisier and Scheele discovered oxygen and its multi-faceted properties. The ‘Oxygen Paradox’ emerged as oxygen or dioxygen, apart from sustaining aerobic life, also possesses a dark side of its own by being toxic to such organisms. Plants and animals survive only for a few days at oxygen concentrations greater than its atmospheric level of 20.9%. Joseph Priestley, in 1775, elegantly put that “we might live out too fast, and the animal powers be too soon exhausted, in this pure kind of air. A moralist, at least, may say that the air which nature has provided for

us is as good as we deserve," (Fridovich 1998; Davies 2000; Freeman 2000; McCord 2000).

Fenton's work, in 1890's, on iron/peroxide chemistry and the recognition that reactive oxygen species (ROS) could introduce reactive diols and carbonyl groups into biomolecules, spawned a whole new field known as Fenton chemistry. Haber, from 1931-34, was responsible for discovery of hydroxyl radical ($OH^{\bullet}$) and the metal catalyzed peroxide decomposition reaction known as the Haber-Weiss reaction. Based on quantum mechanics theory, Pauling (Nobel Prize in Chemistry in 1954 and Nobel Peace Prize in 1962) predicted the existence of superoxide ($O_2^{\bullet -}$) as early as 1933. However, it was only in 1954 that the first significant scientific contribution towards the 'oxygen paradox' was made in the form of a seminal paper by Gershman and Gilbert. They proposed that the lethal effects of ionizing radiation might be ascribed to oxygen free radicals that gave birth to the burgeoning field of free radical biology. In the same year, Commoner presented the first proof that free radicals exist in biological organisms and are either proteinaceous or associated with biopolymers (see reviews by Freeman 2000; Gutteridge and Halliwell 2000; Hensley and Floyd 2002).

In the period 1951-56, Chance worked on the ability of catalase to reduce hydrogen peroxide (H_2O_2) and showed that it can be produced by respiring mitochondria. Based on these observations, Harman in 1956 proposed the classical hypothesis known as the "Free Radical Theory of Aging (FRTA)", which has just turned 50. FRTA states that aging is a result of a) progressive deterioration of physiological function due to free radical reactions initiated in mitochondria at an increasing rate with age and b) the life-span is determined by the rate of free radical damage to the mitochondria, thus giving rise to the "Mitochondrial Theory of Aging (MTA)". The anti-oxidant enzyme copper,zinc-associated superoxide dismutase (Cu,Zn-SOD), discovered by McCord and Fridovich in 1969, followed by Halliwell's report in 1973, marked the dawn of anti-oxidant biology. These studies firmly established that oxygen can biologically be reduced to a diffusible free radical superoxide that can further generate the

toxic and highly reactive hydroxyl radicals, and that all aerobic organisms survive in such harsh environment owing to their arsenal of antioxidant defenses. Later in 1973, Babior's group showed the first physiological role of free radicals in host defense as well as their pathological role in inflammation (Beckman and Ames 1998; Fridovich 1998; Freeman 2000; Gutteridge and Halliwell 2000; Hensley and Floyd 2002; Harman 2003; Harman and Harman 2003).

Environmental sources of free radicals are ozone (O_3), γ-radiation, ultra-violet (UV) radiation, car exhaust, cigarette smoke, industrial contaminants, narcotic drugs, anesthetizing gases, xenobiotics (pesticides, herbicides), chemicals such as alcohol, mustard gas and lastly human diet itself. Major sources of endogenous free radicals in mammals include the respiratory chain, monoamine oxidase, xanthine oxidase, NADPH oxidase, myeloperoxidase, cycloxygenases, cytochrome P-450 system, nitric oxide synthases (NOS) and non-enzymatic reactions of oxygen (Droge 2002; Kohen and Nyska 2002; Andreyev et al. 2005).

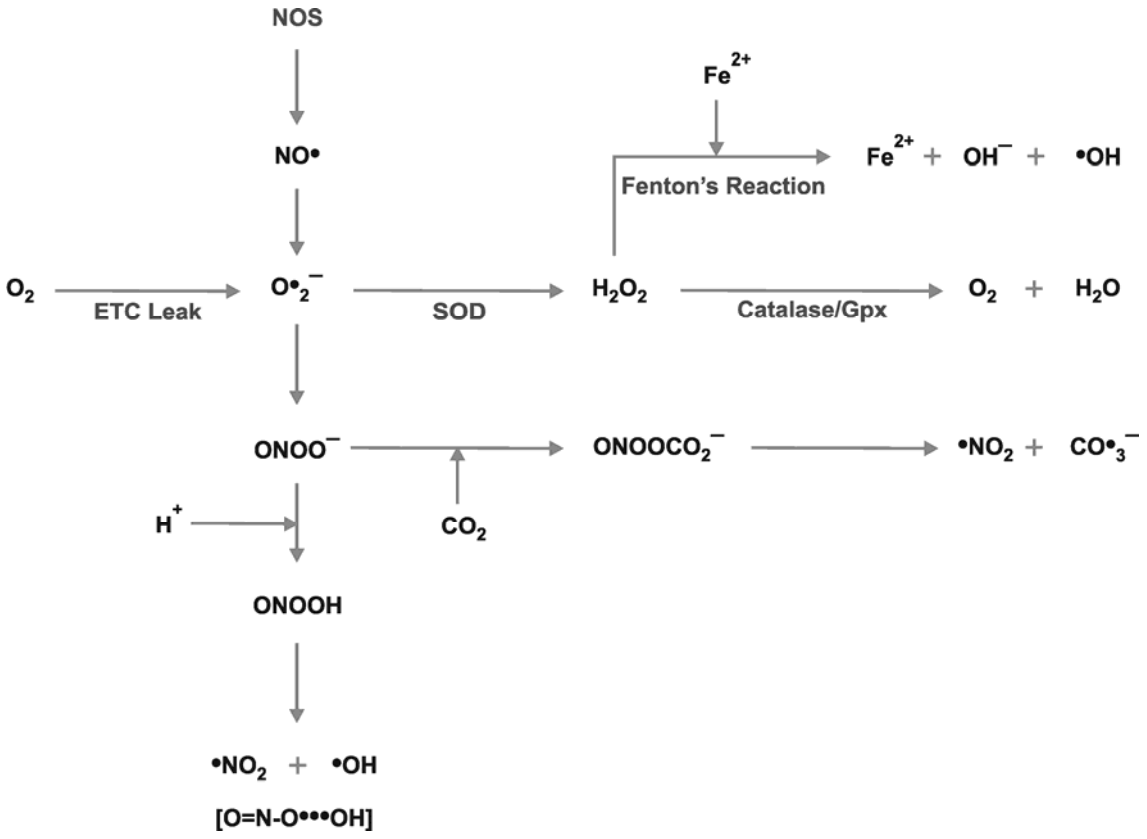
The reactive molecules can be further divided into radicals and non-radical species. Radicals are atoms or molecules containing an unpaired electron, such as nitric oxide radical (NO•), superoxide radical ($O_2^{\bullet -}$), hydroxyl radical (•OH), peroxyl (ROO•) and alkoxyl radicals (RO•), making them highly reactive. There are also other reactive non-radical species such as peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), organic peroxides, aldehydes and ozone (O_3). Their reactivity renders them with short half-lives that can range from nano-seconds to minutes. Hydroxyl radical (•OH) is the most reactive (10^{-9} sec) and therefore attacks any macro-molecules in the immediate vicinity, while nitric oxide (NO•) due to its relatively longer half-life (1-10 sec) can cross membranes and cause damage at a site distant from its origin, whereas the fate of H_2O_2 and $O_2^{\bullet -}$ is only limited by the presence of their decomposition enzymes. This makes them unique molecular signaling switches that can mediate reversible coupled oxidation-reduction reactions of factors involved in

gene and enzyme regulation (Bergendi et al. 1999; McCord 2000; Kohen and Nyska 2002).

Several components of mitochondria are known to be involved in the generation of free radicals as byproducts, mainly in the form of superoxide (around 2% of available oxygen or dioxygen). Oxygen in its ground state contains two unpaired electrons with the same spin and hence exists as a relatively unreactive diradical, which upon partial reduction results in the more reactive superoxide. Normally, the superoxide is converted into hydrogen peroxide (H_2O_2) and oxygen by manganese-superoxide dismutase (Mn-SOD). However, superoxide can convert Fe^{3+} to Fe^{2+} known as the Haber-Weiss reaction. Fe^{2+} in turn can react with H_2O_2 to give Fe^{3+} and the highly reactive OH• radicals, known as the Fenton reaction. H_2O_2 is usually decomposed by catalase or glutathione peroxidase (GPx) into water (Fridovich 1998; Pedersen 1999; McCord 2000; Kohen and Nyska 2002; Andreyev et al. 2005; Scandalios 2005). Nitric oxide (NO•), produced by NOS, can react with superoxide in a diffusion controlled reaction to produce peroxynitrite ($ONOO^-$) (Beckman and Koppenol 1996; Radi et al. 2001). This can further react with carbon dioxide (CO_2), another gaseous messenger, forming nitrosoperoxo carbonate ($ONOOCO_2^-$) that decomposes into nitrogen dioxide ($•NO_2$) and carbonate ($CO_3^{•-}$). Instead, $ONOO^-$ can also gain a proton forming peroxynitrous acid (ONOOH). This in turn can decompose into nitrogen dioxide and hydroxyl radicals which are highly unstable and hence possess a greater potential to cause damage (Bolanos et al. 1997; Bergendi et al. 1999; Radi et al. 2001; Kohen and Nyska 2002).

Figure 1.5: Illustration of the interplay between Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). About 2% of molecular oxygen undergoes partial reduction in the mitochondrial electron transport chain resulting in the more reactive superoxide ($O_2^{\bullet -}$). Normally, the superoxide is converted into hydrogen peroxide (H_2O_2) and oxygen by manganese-superoxide dismutase (Mn-SOD). However, superoxide can convert Fe^{3+} to Fe^{2+} known as the Haber-Weiss reaction. Fe^{2+} in turn can react with H_2O_2 to give Fe^{3+} and the highly reactive •OH radicals, known as the Fenton reaction. H_2O_2 is usually decomposed by catalase or glutathione peroxidase (GPx) into water. Nitric oxide (NO•), produced by NOS, can react with superoxide in a diffusion controlled reaction to produce peroxynitrite ($ONOO^-$). This can further react with carbon dioxide (CO_2) forming nitrosoperoxo carbonate ($ONOOCO_2^-$) that decomposes into nitrogen dioxide ($\bullet NO_2$) and carbonate ($CO_3^{\bullet -}$). Instead, $ONOO^-$ can also gain a proton forming peroxynitrous acid (ONOOH). This in turn can decompose into nitrogen dioxide and hydroxyl radicals both of which are highly unstable and can cause greater damage.

Figure 1.5: Illustration of the interplay between Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS).



Owing to the discovery of NO• and its physiological role, a paradigm shift happened in the field of free radical biology. Free radicals were now looked, under new light, as being regulated physiological signaling molecules rather than damage-causing stochastic entities. This is evident in the roles free radicals play in regulating gene expression, cell replication, differentiation, host-defense (via oxidative burst), cell adhesion, and apoptosis by acting as second messengers in signal transduction pathways. Low levels of oxidants in fact are known to induce cells to proliferate, moderate oxidative environment is shown to induce transient growth arrest, upregulation of anti-oxidant enzymes and stress response proteins, to adapt to the present and cope with future encounters with oxidants (Davies 2000; Droge 2002; Kohen and Nyska 2002; Scandalios 2005).

A classic case of redox signaling is the oxidative burst by immune cells. Activated macrophages and neutrophils can produce large amounts of $O_2^{\bullet -}$ and HOCl, upon activation of NADPH oxidase and myeloperoxidase respectively, induced by lipopolysaccharide (LPS) and pro-inflammatory cytokines such as interferon-γ (IFN-γ), interleukin-8 (IL-8) and interleukin-1β (IL-1β). Activation of NADPH oxidase is controlled by G proteins belonging to rac family. The massive production of antimicrobial and tumoricidal ROS in an inflammatory environment is called the “oxidative burst” and plays an important role as a first line of defense against environmental pathogens (Droge 2002; Babior 2004).

Hydrogen peroxide (H_2O_2) has been shown to oxidatively modify and inhibit protein tyrosine phosphatases, thus increasing net activity of protein tyrosine kinases and thereby altering activities of epidermal growth factor (EGF), insulin receptor kinase (IRK). Hydrogen peroxide (H_2O_2), among others, is also shown to activate protein kinase C (PKC), Src family protein tyrosine kinase, mitogen-activated protein kinases (MAPK) members such as extracellular signal-regulated kinase 1 (ERK-1) and ERK-2, c-Jun NH2-terminal kinase (JNK) and p38, transcription of activator protein 1 (AP-1) consisting of c-Jun and c-Fos. Finally, ROS can also activate NF-κB, the first transcription factor responding directly to oxidative stress, by promoting degradation of its partner I-κB by the proteasome. ROS can also alter the binding sites on DNA, of transcription factors

such as NF-κB and AP-1, thereby affecting the expression of specific genes. The redox signaling is further modulated by the redox state of glutathione inside the cell. However, i*n vivo*, cells function normally in a reducing environment and their function is invariably altered when the environment is increasingly oxidized, leading to oxidative stress (Davies 2000; Droge 2002; Kohen and Nyska 2002; Scandalios 2005).

With the advent of aerobic life, an arsenal of antioxidants and antioxidant enzymes evolved to protect the cells from oxidative stress and damage. Antioxidants are defined as substances that are able, at relatively low concentrations, to compete with other oxidizable substrates and, thus significantly delay or inhibit the oxidation of these substrates. Mammalian cells exist *in vivo* at 2-4% oxygen tension known as tissue normoxia, compared to 21% in the surrounding environment, creating an oxygen tension gradient. Therefore, multiple layers of defenses will be put forth as per the need, to maintain redox homeostasis and minimize free radical–induced damage, that include low-molecular-weight antioxidants (LMWA) such as ascorbate, tocopherols, glutathione, melatonin, histidine dipeptides, β-carotene, ubiquinone and uric acid (produced by xanthine dehydrogenase). Ascorbate (Vitamin C), a water-soluble antioxidant, is available to humans only through diet. It mediates its antioxidant effects by donating two electrons to ROS, such as $O_2\bullet^-$, •OH, ROO•, HOCl and ONOOH, and removing them. Tocopherols (Vitamin E) stabilize cellular membranes by donating hydrogens lost by fatty acid radicals, thus acting as chain-breaking antioxidants. A similar mechanism is utilized by melatonin, hormone synthesized by the pineal gland. Glutathione (GSH), a reduced tripeptide (glutamic acid-cysteine-glycine) present in milli-molar concentrations inside the cell, is involved as a cofactor for peroxidase donating electrons for decomposition of H_2O_2 as well as a chelator of Cu^+ preventing the Haber-Weiss reaction. When oxidized, GSH is converted to GSSG formed by joining of two GSH molecules by a disulfide bridge. It can directly interact with superoxide radical ($O_2\bullet^-$), hydroxyl radical (•OH), peroxyl (ROO•), alkoxyl radicals (RO•) and hypochlorous acid (HOCl) forming a glutathione radical that can be regenerated

to its reduced form. Histidine dipeptides, synthesized in the brain and skeletal muscles, can also directly exert a similar action as GSH. Uric acid, in the form of urate, can react with •OH, ROO• as well as $ONOO^-$ to prevent protein nitration (Kohen and Nyska 2002; Scandalios 2005).

Endogenous anti-oxidant enzymes include peroxidases (e.g., peroxisomal catalase and cytosolic glutathione peroxidase), DT diaphorase or quinone reductase, superoxide dismutases (cytosolic Cu,Zn-SOD, extracellular EC-SOD and mitochondrial Mn-SOD), cytosolic Fe-binding ferritin and plasma Cu-binding cerruloplasmin. Apart from above, there are damage repair systems that can be divided into direct and indirect repair (Davies 2000; Kohen and Nyska 2002; Andreyev et al. 2005). However, the antioxidant defenses are not adequate and when in excess, free radicals can react with macromolecules, such as proteins, lipid membranes and DNA, in a self-perpetuating manner thereby disrupting the redox homeostasis. The extent of damage is based on their reactivities and half-lives, apart from their intracellular source and the surrounding anti-oxidant environment (Bergendi et al. 1999; Kohen and Nyska 2002; Scandalios 2005).

Lipid peroxidation occurs upon oxidative modification of membrane phospholipids. It occurs in 3 stages- a) chain initiation b) chain propagation and c) chain termination. Initiation occurs by the abstraction of a hydrogen atom (from carbon of a methylene group) in an unsaturated fatty acyl chain. Following hydrogen abstraction, the remaining fatty acid radical retains 1 electron and is stabilized by rearrangement of the molecular structure to form a conjugated diene. In an aerobic environment, oxygen will add to the fatty acid at the carbon centered lipid radical (L•) to give rise to a lipid peroxyl radical (LOO•). Once initiated, LOO• can further propagate the peroxidation chain reaction by abstracting a hydrogen atom from other vicinal unsaturated fatty acids. The resulting lipid hydroperoxide (LOOH) can easily decompose into several reactive species including: lipid alkoxyl radicals (LO•), aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), alkanes, lipid epoxides, and alcohols. Chain termination occurs upon interaction of LOO• with another radical or an antioxidant. Peroxidized membranes become rigid, lose selective

permeability, and under extreme conditions lose their structural and functional integrity. Lipid peroxidation is also thought to lead to formation of DNA adducts as well as inhibition of enzyme function. Oxidized lipid layers are subject to the actions of phospholipase A_2, which prevents the propagation reactions by removing the membrane hydroperoxides, or glutathione peroxidase, which acts by reducing fatty acid hydroperoxides to their corresponding hydroxyl fatty acids (Davies 2000; Kohen and Nyska 2002).

Protein oxidation refers to oxidative modification of a protein, usually in the form of highly reactive carbonyl groups (aldehydes and ketones). Cysteine (Cys) and methionine (Met), owing to their susceptible sulfur atoms, are two amino acids that are most prone to oxidative attacks. Oxidation of Cys leads to intra-molecular disulfide bonds, inter-molecular disulfides (with glutathione etc). Methionine (Met), on the other hand, undergoes oxidation to give Met sulfoxide. Both Cys and Met can undergo oxidation directly by γ–radiation through the highly reactive hydroxyl (•OH) radicals, discovered by Stadtman's group. Alternatively, their oxidation can be catalyzed by protein-bound transition metals iron (Fe^{2+}) and copper (Cu^{+}) as shown by Levine's group or by $ONOO^-$, $O_2^{\bullet -}$, •OH and HOCl. Lysine (Lys), histidine (His) and Cys are susceptible to form lipid-peroxidation adducts. Disulfide reductase and methionine sulfoxide reductase directly mediate re-reduction of oxidized sulfhydryl groups on proteins. 3-nitrotyrosine (3-NT) is considered to be a specific marker of $ONOO^-$ mediated oxidation. Other amino acids subject to oxidation include arginine (Arg), proline (Pro), threonine (Thr) and tryptophan (Trp), resulting in aldehydes or ketones. Oxidative modification of key residues in proteins could result in loss or gain of function of enzymes or structural proteins (Shacter 2000; Stadtman and Levine 2003).

Intracellular proteolysis can be carried out by mitochondrial proteases, lysosomal proteases, calcium-activated calpains, or the ubiquitin-proteasome system found in the cytosol, nucleus and ER of cells. Most of the oxidized proteins are selectively degraded by the proteasome, which is involved in the normal turn-over of, regulatory proteins involved in cell cycle, cell surface

receptors and ion channels, as well as antigen presentation and processing. An enzyme cascade consisting of ubiquitin ligases tag the proteins on their lysine residues to be degraded with poly-ubiquitin molecules, except when the proteins are oxidatively modified, and process them to the proteasome. The removal of the ubiquitin molecules before degradation requires ATP. (This work on ubiquitin-mediated proteolysis, by Ciechanover, Hershko and Rose, was awarded with the Nobel Prize in Chemistry in 2004). Oxidized proteins, as opposed to native proteins, are recognized by their hydrophobic surface patches, formed by partial unfolding and exposure of hydrophobic amino acid residues due to oxidation. Mitochondria contain three ATP-dependent proteases namely Lon, Clp-like and AAA. Lon protease is shown to be responsible for degradation of oxidatively modified proteins in the mitochondrial matrix. It is also shown to bind mtDNA and act as a chaperone protein. However, during oxidative stress, there may be an overload on the proteasome system for degradation as well as the proteasome itself might be oxidatively modified and damaged, leading to formation of insoluble and stable aggregates of misfolded/damaged proteins, such as lipofuscin, both inside the cell and extracellularly (Davies 2000; Grune et al. 2003; Stadtman and Levine 2003; Grune et al. 2004; Ciechanover 2006; Friguet 2006).

De-oxyribo nucleic acid (DNA) in the nucleus contains the genetic code necessary for cell survival, growth and cell division, in other words life. However, DNA is rather susceptible to free-radical mediated damage, even more so the case with mitochondrial DNA (mtDNA) due to its close proximity to sites of ROS generation and the lack of protective histones. Damage occurs in the form of modification of DNA bases, single- and double-strand breaks, loss of purines and DNA-protein cross-linkage. Most of the damage to DNA such as 8-hydroxy-2-deoxyguanosine (8-OHdG), 8-hydroxyadenine, thymine peroxide and thymine glycol is caused by •OH radicals (Kohen and Nyska 2002). Modified DNA can be repaired by DNA glycosylases, endonucleases, polymerases and ligases. However, if insufficient, oxidized DNA results in mutations leading to transcription and translation of mutated proteins that in turn may have lost function or gained

an undesirable one. Such mutations in DNA can also be passed onto the progeny through the germ-line. The resulting oxidative stress has been implicated in cancer, diabetes, cardiovascular diseases, chronic inflammation, aging and neurodegenerative disorders (Dykens 1994; Bolanos et al. 1997; Droge 2002; Kohen and Nyska 2002; Wallace 2005).

Cell Death

The importance of programmed cell death during development by Brenner, Horvitz and Sulston was recognized and accorded a Nobel Prize in Physiology/Medicine in 2002. Cell death signals invariably converge on mitochondria which then commit the cell to apoptosis and/or necrosis to maintain tissue homeostasis (Brown et al. 1999; Kroemer and Reed 2000; Lemasters 2005). It should be noted that, although apoptosis and necrosis are considered in an oversimplified manner to be distinct forms of cell death, there is evidence for their coexistence. Both forms of cell death are characterized by mitochondrial permeability transition, defined as the loss of trans-membrane potential across the mitochondrial inner membrane. The MPT pore itself is described as voltage-dependent, cyclosporine-sensitive, high conductance channel involving the inner mitochondrial membrane. One of the strongest inducers of MPT is Ca^{2+} that can be competitively inhibited by Mg^{2+}, manganese (Mn^{2+}) and Pi. Matrix pH below and above 7.4 also induces MPT. MPT also causes loss of pyridine nucleotides needed for respiration and membrane depolarization leads to inhibition of ATP synthesis. The route of cell death is dependent upon severity of the insult and intracellular levels of ATP, wherein apoptosis requires ATP and necrosis occurs upon depletion of cellular ATP (Leist et al. 1999; Nicotera and Melino 2004; Bernardi et al. 2006; Zong and Thompson 2006), with exceptions (Chiarugi 2005).

Apoptosis, coined by Kerr and colleagues in 1972 and synonymously used with “programmed cell death” and “cell suicide”, is derived from Greek and stands for “falling off”, referring to an orchestrated physiological process of

removing individual components with minimal damage to the organism (Kerr 2002). All eukaryotic cells are equipped with genetic machinery, of varying similarities conserved evolutionarily, for undergoing apoptosis. Apoptosis is necessary for gametogenesis, embryogenesis and hematopoiesis, cardiac, immune and neuronal development as well normal adult tissue turnover, owing to the property of apoptotic cells not causing inflammation or tissue scarring. This not only ensures stable cell populations but is also important for host survival during invasions by pathogens (Kerr et al. 1972; Ranger et al. 2001; McHugh and Turina 2006). On the other hand, inhibition of apoptosis can cause a wide variety of pathological conditions such as cancer and auto-immunity whereas chronic activation of apoptosis is implicated in aging (Pollack et al. 2002; Ziegler and Groscurth 2004; Lemasters 2005) and neurodegeneration (Ranger et al. 2001; Ameisen 2002; Fadeel and Orrenius 2005).

Apoptosis is an ATP-dependent process and typically characterized by chromatin condensation and inter-nucleosomal DNA degradation termed as pyknosis, cell shrinkage, formation of numerous small surface blebs, externalization of phosphatidyl serine (PS) on the plasma membrane, resulting in condensed apoptotic bodies tagged for phagocytosis (Ziegler and Groscurth 2004; Lemasters 2005). Depending on the source of signal, cells can undergo apoptosis either by the extrinsic pathway, for normal tissue homeostasis, or alternately the intrinsic pathway initiated by mitochondria due to cues from the nucleus or other noxious stimuli (Fadeel and Orrenius 2005).

Both the extrinsic and intrinsic pathways involve caspase activation. Caspases are constitutively expressed in mammals as inactive zymogens and proteolytically activated to form active tetramers. They are cysteine proteases with preference to substrate cleavage after aspartate residues. They can be divided into initiator (2, 8, 9, 10 and 12), effector (3, 6 and 7) and inflammation (1, 5 and 11) caspases. The initiator caspases contain share conserved motifs known as caspase recruitment domain (CARD), death effector domain (DED) and the catalytic domain, while the effector caspases possess a short pro-domain. The initiator caspases are activated by an autocatalytic mechanism

whereas they are required for activation of the effector caspases. Effector caspases are able to directly cleave their substrates that include proteins involved in scaffolding of the cytoplasm and nucleus, signal transduction and transcription-regulatory proteins, cell cycle components, DNA replication and repair proteins, and apoptosis inhibitors to form a positive feedback loop (Ranger et al. 2001; Nicotera and Melino 2004; Artal-Sanz and Tavernarakis 2005; Fadeel and Orrenius 2005).

The extrinsic pathway (death-receptor signaling) is triggered upon binding of ligands to their receptors such as Fas-ligand/Fas, TNFα/TNFR1, their association with adapter proteins (FADD and TRADD respectively) forming a death-inducing signaling complex (DISC) and subsequent activation of initiator caspases such as caspase-8. It in turn can proteolytically activate execution caspases - caspase-3. In some cells, caspase-8 further cleaves pro-apoptotic Bid, resulting in its translocation to mitochondria and subsequent cytochrome c release in order to generate sufficient caspase-3 activity (Ranger et al. 2001; Nicotera and Melino 2004; Fadeel and Orrenius 2005).

On the other hand, nuclear DNA damage, kinase inhibition, trophic factor deprivation, UV and calcium/oxidative stress act as triggers of the intrinsic pathway of apoptosis. This causes, the pro-apoptotic proteins of Bcl2 family, Bak and Bax to translocate from cytosol to mitochondria and interact with anti-apoptotic $Bclx_L$ & Bcl2 and induce mitochondrial membrane permeability transition (MPT). This results in the formation of a MPT pore (MPTP), causing membrane depolarization and inhibition of respiration. Some reports suggest that the loss in membrane potential causes more ions to flow into the matrix followed by influx of water. This leads to swelling of the matrix and rupture of mitochondrial outer membrane. Other reports indicate that the pro-apoptotic proteins are released through the formation of a pore, comprising of Bax and other proteins such as peripheral benzodiazepine receptor (PBR), hexokinase (HK), and creatine kinase (CK), the exact composition and mechanisms of which are still unclear (Crompton 2003; Hajnoczky et al. 2003; Kim et al. 2003; Armstrong 2006; Bernardi et al. 2006). This results in release of cytochrome c,

second mitocondria-derived activator of caspases/ direct IAP-binding protein with low pI (Smac/Diablo), procaspases-2,3,8 and 9, apoptotic protease-activating factor 1 (Apaf-1), apoptosis-inducing factor (AIF), Endonuclease G (Endo G) and possibly high temperature requirement protein A2 (HtrA2/Omi) (Ranger et al. 2001; Parone et al. 2002; Armstrong 2006).

Apaf-1 binds with cytochrome c, in the presence of ATP, and recruits procaspase-9, forming a complex called the “apoptosome”. The now activated caspase-9 activates caspase-3. JNK, a kinase, also utilizes ATP for activation of pro-apoptotic proteins such as Bid and Bad, while inactivating anti-apoptotic proteins such as Bcl-2. Smac/Diablo, for its part, binds with the regulatory inhibitors of apoptosis proteins (IAP), resulting in disinhibition of both the initiator and effector caspases. On the other hand, in a caspase-independent manner, AIF and Endo G translocate from cytosol to nucleus and induce chromatin condensation and inter-nucleosomal DNA fragmentation respectively (Ranger et al. 2001; Lemasters 2005; Armstrong 2006).

In contrast to apoptosis, necrosis is not programmed and occurs when cellular ATP is depleted, and is usually initiated by Ca^{2+} and ROS. Necrosis is characterized by electron-lucent vacuolation of cytoplasm, mitochondrial swelling, dilatation of ER and loss of PM integrity and subsequent rupture. MPT, in the case of necrosis, involves permeability of the inner mitochondrial membrane, resulting in a regulated high-conductance pore formed by voltage-dependent anion channel (VDAC) from the outer membrane, adenine nucleotide translocator (ANT) from the inner membrane, cyclophilin D (CypD) from the matrix, or a more unregulated pore formed due to disulfide bridges among proteins of the inner mitochondrial membrane, the mechanisms of which are yet unclear. This leads to release of various matrix components into the cytosol that can be degraded by the proteolytic machinery in the cytosol. Some of them include the calcium-activated calpain proteases that can be divided into μ-calpain (calpain I) and m-calpain (calpain II), based on their micro and millimolar requirements of Ca^{2+} for activation. They exist as heterodimers consisting of a large catalytic sub-unit and a smaller regulatory sub-unit and when activated

bring about degradation of cytoskeletal proteins. Lack of ATP reverses some of the PM pumps resulting in influx of ions and water into the cell resulting in its swelling and extrusion of its contents into the extracellular space thereby eliciting an inflammatory response (Leist et al. 1999; Crompton 2003; Kim et al. 2003; Nicotera and Melino 2004; Artal-Sanz and Tavernarakis 2005; Wallace 2005; Armstrong 2006; Bernardi et al. 2006; Zong and Thompson 2006).

When the defective or damaged mitochondria reach a certain threshold they are tagged for degradation by autophagy, more specifically called mitophagy. This is preceded by loss of mitochondrial membrane potential and release of cytochrome c and other pro-apoptotic proteins and usually followed by cell death. Mitophagy occurs in autophagolysosomes (autolysosomes) formed by fusion of lysosomes (the work is credited to de Duve who won the Nobel Prize in Physiology/Medicine in 1974) and autophagic vacuoles containing the mitochondria. Autolysosmes contain a variety of catabolic hydrolases (phosphatases, nucleases, glycosidases, proteases, peptidases, sulphatases and lipases) that operate at an acidic pH (4-5) and can digest the lysosomal contents for reuse. Lysosomal cathepsin proteases can be divided into aspartyl (cathepsin-D) and cysteine (cathepsin-B, H and L), and can cause non-specific cleavage of proteins in lysosmes and sometimes in the cytosol. They can also activate pro-apoptotic Bid leading to mitochondrial membrane permeabilization and release of cytochrome c (Artal-Sanz and Tavernarakis 2005; Kroemer and Jaattela 2005; Lemasters 2005). However, it is unclear at present if mitophagy is protective by providing energy generated from catabolic degradation of damaged mitochondria or if it hastens cell death by removing the major sources of energy from the cell (Klionsky and Emr 2000; Tolkovsky et al. 2002; Rodriguez-Enriquez et al. 2004; Golstein and Kroemer 2005; Kundu and Thompson 2005). Finally, there appears to be cross-talk between caspases, calpains and cathepsins leading to a continuum between apoptotic and necrotic forms of cell death.

Aging: A Full Circle

Life of an organism is a function of its resistance to entropy i.e., the tendency of complex systems to move towards disorder and decay over time. Hence, life requires and utilizes energy, obtained from the available metabolic resources, to sustain a delicate balance among the competing processes namely growth, maintenance/repair and reproduction (Kirkwood and Austad 2000; Kirkwood 2005; Holliday 2006). Aging can be defined as an irreversible alteration in the intrinsic homeostasis of an organism, affected by its genetic make-up, environmental stresses and other stochastic events, leading to a) diminishing physiological capacity, b) decreased adaptability, c) a decline in fertility, d) increased vulnerability to disease and e) an exponential increase in mortality after maturation. Aging particularly increases the risk of occurrence of diseases, such as cancer, diabetes, cardio-vascular conditions as well as neurodegenerative conditions such as Alzheimer's disease (AD), Parkinson's disease (PD) and dementia, resulting ultimately in death (Troen 2003; Kirkwood 2005; Wallace 2005).

Longevity of a population can be measured by two different parameters – a) average life expectancy (ALE) and b) maximum lifespan (MLS). Life expectancy is the average/median age at which 50% of the population survives whereas maximum lifespan is the age of the longest-living member of that population. Improved nutrition, better sanitation and advances in health care have removed the humans from the pressures of natural selection and dramatically increased the ALE of humans over the past century, whereas the MLS in humans stands at 122 years indicating an underlying genetic component. This is particularly important in the context of United States where life expectancy at birth has increased from being less than 50 years to more than 76 years. It is projected that by the year 2030 the number of individuals aged 65 years and older will be around 70 million and those aged 85 years and older is estimated to be around 19 million by the year 2050. The ethical, medical and economic ramifications of such an aging population can already be felt by the society today.

The focus of human aging research is not so much on reversing aging but geared towards gaining insights of the underlying mechanisms to modulate aging and thereby ensuring a higher quality of life in the later years (Harman 2003; Troen 2003).

Aging is not typically observed in natural populations as they do not survive extrinsic hazards such as starvation, predators, disease and other natural calamities. The main purpose of such organisms is to reach adulthood and be able to reproduce and generate progeny. The same animals when placed in protected environment with adequate resources are likely to live longer and exhibit an aging phenotype. While longevity refers to the time-frame of the organism, aging refers to the deleterious process occurring during that time. There exists an inverse relation between reproduction and longevity i.e. organisms with larger progeny have reduced life spans and vice versa. This is dependent on the allocation of limited resources to reproduction as opposed to somatic maintenance, as stated by the complementary 'mutation accumulation', 'antagonistic pleiotrophy' and the 'disposable soma' theories of aging that attempt to explain *why* aging occurs evolutionarily (Kirkwood and Austad 2000; Kirkwood 2005; Holliday 2006).

Owing to the evolutionary counter-intuitiveness and inherent complexity, a unifying theory is precluded from successfully elucidating the phenomenon of aging. Therefore, various theories have been put forth to explain *how* aging occurs that can be broadly divided into programmed (ex: longevity genes) and stochastic (ex: oxidative stress). While such mechanistic theories are not mutually exclusive and attempt to gain attention in the competing 'cause vs. effect' discussions, the 'Mitochondrial Theory of Aging (MTA)' offers to explain both the stochastic and genetic components associated with aging (Harman 2003; Troen 2003; Kirkwood 2005; Wallace 2005).

Aging of the brain is characterized by mild cognitive impairment (MCI) and impairment of sensory and motor functions that has the potential to develop into neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's (PD). The decline or loss of cognitive function with aging is not due to universal loss of

neuronal cells per se, as previously thought, but primarily due to loss of synaptic function in distinct cell populations. Selective regions in the cortex and hippocampus are more susceptible to synaptic reduction, dendritic degeneration and/or neuronal cell loss thereby affecting learning and memory (Uylings and de Brabander 2002).

The brain is a unique organ in that it consists of mostly post-mitotic cells, has a heterogeneous composition of cell types (neurons, astrocytes, glia etc), has distinct structural (cortex, hippocampus, striatum etc) regions and functional (motor vs. sensory vs. memory) areas. It has low levels of stored glycogen, almost exclusively utilizes glucose as its energy source and consumes about 20% of total body oxygen even though it constitutes only 2% of the body weight. In addition, it contains high amounts of unsaturated fatty acids along with high iron content and found to be low in antioxidant defenses. All the above reasons make the brain a prime target for oxidative stress that is believed to increase with aging. In fact, increased protein oxidation, lipid peroxidation and DNA modifications, all markers of oxidative damage, were observed with aging that correlate well with cognitive dysfunction. There is also a strong negative correlation between energy metabolism and lifespan in mammals, lending credibility to the free radical theory of aging (Levine and Stadtman 2001; Floyd and Hensley 2002; Dufour and Larsson 2004; Poon et al. 2004; Stadtman 2006).

Various groups have presented divergent reports on the distribution, expression, activity of different NOS isoforms in the brain and resultant protein modifications. Contradictory reports have emerged regarding the role of NO• and the extent of RNS mediated damage with aging measured by levels of 3-NT in different regions of the brain. However, there are no reports to date characterizing mtNOS as a function of age in the brain. None of these studies addressed the source of 3-NT measured and could plausibly account for the discrepancies (Bolanos et al. 1997; Drew and Leeuwenburgh 2002; Calabrese et al. 2006).

According to the Ca^{2+} hypothesis of brain aging, there is dysregulation of calcium homeostasis in the brain with aging that has a detrimental cascade effect

on Ca^{2+}-dependent processes thereby impairing brain synaptic function. This is evident from the increased vulnerability to excitotoxicity with age, increased numbers and activity of Ca^{2+}-dependent channels, decreased capacity to recover after stimulation (although intracellular Ca^{2+} levels at rest do not change with age), increased Ca^{2+} influx into mitochondria or finally due to enhanced mitochondrial permeability transition pore (MPTP) activation with aging. Such Ca^{2+} dysregulation may lead to the undesirable consequence of impaired memory and cognition (Brown et al. 2004; Toescu and Verkhratsky 2004; Toescu et al. 2004; Kelly et al. 2006).

Mitochondria occupy the center stage in aging and neurodegeneration owing to their capacity for- a) providing cellular ATP, b) buffering calcium, c) being a major source and target of ROS/RNS and finally d) being initiators of cell death pathways (Beal 2005). Therefore, mitochondria provide the converging point to successfully combine the 'calcium dyshomeostasis hypothesis' (Toescu et al. 2004) with the 'free radical theory' (Miquel 2002; Wei and Lee 2002; Harman 2003; Wallace 2005; Lenaz et al. 2006) of brain aging into a synergistic Mitochondrial Theory of Aging (MTA). The MTA postulates that mitochondrial dysfunction, due to a stochastic and exponential rise in mtDNA mutations leading to defective ETC complexes causing a reduction in ATP generation/levels and increased oxidative stress leading to irreversible alterations in mitochondrial structure and function, is central to the decline in function observed with aging (Miquel 2002; Wei and Lee 2002; Harman 2003; Wallace 2005; Lenaz et al. 2006).

It has been documented that, with age, brain accumulates mtDNA deletions, rearrangements and point mutations resulting in a mixed population of around 1% of total mtDNA. Since mtDNA codes for 13 of the subunits of the ETC complexes I, III, IV and V, any mutations in their coding regions will result in synthesis of defective respiratory complexes decreasing their efficiency to carry out respiration. One of the fallouts of this is the increased leakage of electrons from the ETC, generating more free radicals. These are in turn shown to cause oxidative modifications of mtDNA as well as other mitochondrial proteins (ex:

ANT) and enzymes (ex: MnSOD) reducing or inhibiting their activities. Brain mitochondrial membranes have a higher concentration of polyunsaturated lipids and age-related decrease in amounts of cardiolipin which makes them more susceptible to free radical damage and thereby alters their fluidity, protein-lipid interactions and ultimately their structure. Taken together, there is a positive correlation between oxidative damage to mitochondrial components and decline in ATP generation and levels. Moreover, decreases in mitochondrial membrane potential, oxygen consumption and respiratory control ratio (RCR) were also observed with age resulting in a bioenergetic failure of mitochondria. In addition, aged mitochondria were also more susceptible to Ca^{2+} and free radical induced MPT (Bolanos et al. 1997; Toescu et al. 2000; Wei and Lee 2002; Brown et al. 2004; Dufour and Larsson 2004; Liu and Ames 2005; Wallace 2005).

Cell death resulting from mitochondrial dysfunction is not readily evident with normal aging in the brain (Pollack et al. 2002). One of the reasons for this difference could be the intra-cellular trafficking and location (soma vs. synapses) of mitochondria, conditions in their surrounding milieu and their functional status within the neuron (Chang and Reynolds 2006). The cognitive decline with aging is explained by the changes in neuronal structure and loss of neuronal function, resulting from mitochondrial dysfunction, rather than neuronal cell death. However, cell death was indeed observed in other tissues with aging and more pronouncedly in age-related neurodegeneration (Uylings and de Brabander 2002; Sastre et al. 2003; Sullivan 2005; Sullivan and Brown 2005; Mattson 2006).

Summary

Mitochondria occupy a unique position inside the cell, in the sense they are the non-nuclear master regulators of cell's fate. Their primary and most vital function is to provide ATP energy for the normal physiology of the cell, with an unfortunate but an inevitable consequence being generation of free radicals. In addition, they also possess calcium-buffering capacity. The combination of Ca^{2+}

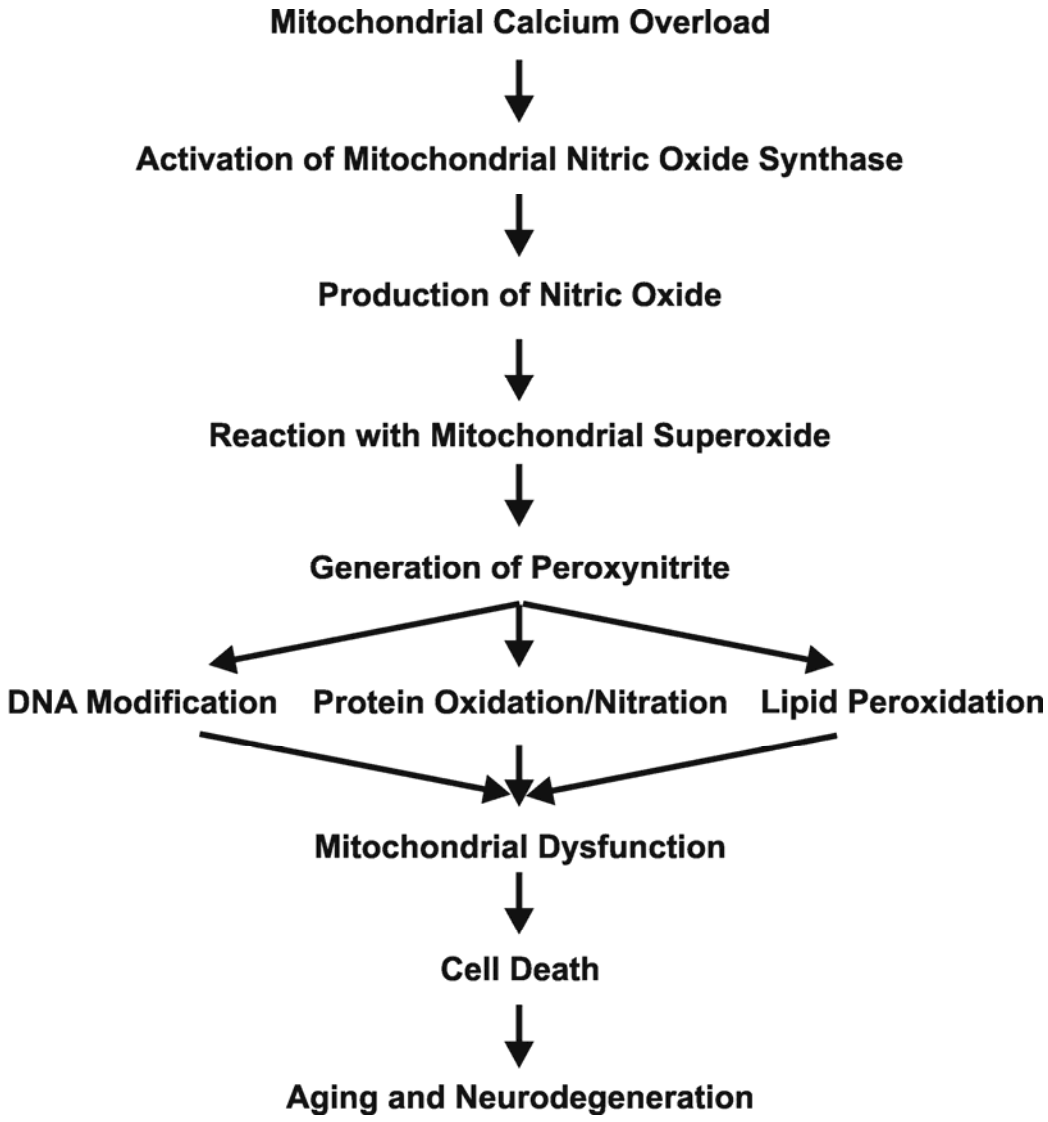
and free radicals, under certain conditions, could lead to oxidative stress. And finally, they also bring about the death of the cell in the form apoptosis/necrosis that could be beneficial as in development or detrimental as in the case of aging, neurodegeneration and other pathologies.

Brain mitochondria in particular are more susceptible to oxidative stress that is greatly modulated by the cellular Ca^{2+} fluxes as implicated in aging and age-related neurodegeneration conditions such as Alzheimer's (AD) and Parkinson's (PD). However, the sources of ROS/RNS, how their levels are altered by calcium and how the resulting oxidative stress is responsible for mitochondrial dysfunction observed with aging are all questions begging further clarification.

It is of critical importance to prevent or ameliorate mitochondrial oxidative stress and preserve mitochondrial bioenergetics in order to maintain cellular homeostasis. Thus, it becomes imperative to understand the underlying causes, mechanisms and consequences of mitochondrial oxidative stress. Therefore, the purpose of this dissertation is to study **if there is cross-talk between calcium and nitric oxide synthase that affects mitochondrial bioenergetics and contributes to oxidative stress in isolated rat brain mitochondria.**

Figure 1.6: Summary of hypothesis. Flowchart of plausible sequence of events resulting in mitochondrial dysfunction implicated in aging and neurodegeneration. Excess cytosolic calcium (Ca^{2+}) is taken up by mitochondria which can activate a number of enzymes in the mitochondria including Nitric oxide Synthase (NOS). This leads to production of nitric oxide (NO•) that can react with superoxide ($O_2^{\bullet -}$) forming peroxynitrite ($ONOO^-$). It inturn can cause oxidative damage to various mitochondrial components namely mtDNA, proteins and lipids. This will alter mitochondrial structure and function resulting in reduced energy generation and/or initiation of cell death cascades. Loss of cell function and cell numbers are implicated in aging and age-related neurodegenerative conditions such as Alzheimer's disease (AD).

Figure 1.6: Summary of hypothesis.



Chapter Two

Cryopreservation of Brain Mitochondria: A Novel Methodology for Functional Studies

Introduction

Mitochondria are essential for aerobic metabolism and ATP production in the cell [for reviews see (Szewczyk and Wojtczak 2002; Watts and Kline 2003)] and play a significant role in cell death as relevant to aging and neurodegenerative diseases (Lemasters et al. 1999; Newmeyer and Ferguson-Miller 2003). Mitochondrial function is typically analyzed using mitochondria freshly isolated from tissues and cells because they yield tightly coupled mitochondria, whereas those from frozen tissue can consist of broken mitochondria and membrane fragments (Lee 1995). This prevents serial usage as well as long-term batch storage or 'banking' of isolated mitochondria for later use in functional studies (Kuznetsov et al. 2003). To overcome such limitations, research has been done to optimize methods to cryopreserve mitochondria without affecting the structural and functional integrity of isolated mitochondria.

There are various natural and synthetic compounds currently being used as cryoprotective additives (CPA) such as alcohols, saccharides, heterocyclic compounds, certain amino-acids and proteins, as well as complex substrates such as yeast extract and skimmed milk. Of the lot, glycerol and dimethyl sulfoxide (DMSO) are the most widely used CPA. DMSO in particular is considered to be an effective cryoprotectant since it is cell-permeable, universal and well-characterized (Hubalek 2003; Santos et al. 2003). While previous studies have demonstrated cryopreservation of isolated mitochondria using DMSO in liver and muscles of rabbits (Araki 1977; Araki 1977), mice (Fishbein and Griffin 1976), rats (Tsvetkov et al. 1985; Tsvetkov et al. 1985; De Loecker et al. 1991; Kuznetsov et al. 2003) and humans (Lee 1995; Kuznetsov et al. 2003),

relatively few studies have directly addressed the effects of cryopreservation on the mitochondria in rat brain (Lee 1995).

In the present study, cortical mitochondria were isolated from adult Sprague-Dawley rats. DMSO (10% v/v) was added to the crude or percoll-purified fraction and assessed for respiration or allowed to cool at a uniform rate of ~ 1ºC/min and stored at -80ºC for a week. The cryopreserved mitochondria were reanimated after a week and assessed for respiration while structural intactness was verified by electron microscopy. The samples used for respiration studies were also used to measure the levels of mitochondrial marker proteins namely COX or COX IV (inner membrane), Cytochrome c (inter-membrane space) and VDAC (outer membrane).

We conclude that DMSO can be successfully used to cryoprotect rat cortical mitochondria which could be extended as a possible strategy for preservation of human brain mitochondria as well. This will considerably expand the range of biochemical, molecular and metabolic studies that can be performed on rat or human post-mortem tissues without the constraints of mitochondrial longevity ex vivo and allow the banking of samples for future comparative studies.

Materials and Methods

Reagents:

Mannitol, sucrose, bovine serum albumin (BSA), ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), hydroxyethyl piperazine-1-ethanesulfonic acid potassium salt (HEPES), potassium phosphate monobasic anhydrous (KH_2PO_4), magnesium chloride ($MgCl_2$), potassium chloride (KCl), malate, pyruvate, adenosine 5'-diphosphate (ADP), digitonin, succinate and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oligomycin A and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) were purchased from Biomol (Plymouth Meeting, PA). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL).

Isolation and Purification of Brain Mitochondria:

All experimental protocols involving animals were approved by the University of Kentucky Animal Use and Care Committee. This protocol contains modifications of previously described procedures (Brown et al. 2004; Sullivan et al. 2004). All procedures were performed on ice throughout the protocol. Depending on the sample size, this protocol requires about 2.5 – 3hrs of total preparation time. Male Sprague-Dawley rats (~250g) were decapitated and the brains were rapidly removed. The cortices were dissected out and placed in an all-glass dounce homogenizer containing five times the volume of isolation buffer with 1mM EGTA (215 mM mannitol, 75mM sucrose, 0.1% BSA, 20mM HEPES, 1mM EGTA and pH is adjusted to 7.2 with KOH). The tissue was homogenized and mitochondria were isolated by differential centrifugation. Briefly, the homogenate was centrifuged at 1300 x g for 3 minutes in an eppendorf microcentrifuge at 4ºC and the supernatant was transferred to new tubes. The loose pellet was resuspended in isolation buffer with EGTA and was centrifuged again at 1300 x g for 3 minutes. The resulting supernatant was transferred to new microcentrifuge tubes and topped off with isolation buffer with EGTA and centrifuged at 13,000 x g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 500 µL of isolation buffer with EGTA. A fraction of the sample was aliquoted for cryopreservation. To obtain maximal yield of total (synaptic and non-synaptic) mitochondria, synaptosomal membranes were disrupted by adding 0.01% Digitonin (from 10X stock) to the sample and incubated on ice for 10 min. To remove the digitonin, the sample was topped off with isolation buffer and centrifuged at 10000 x g for 10 min. The sample was further purified by percoll density gradient centrifugation. Stocks of 40%, 26% and 24% percoll were prepared fresh in isolation buffer with EGTA. 3.5 ml of 26% stock was carefully layered over 3.5 ml of 40% in 13 ml ultra-clear tubes. Equal volume of 24% percoll was added to the sample to get a 12% final concentration and loaded gently on top of the 26% layer. The sample was centrifuged in fixed angle type 50.2 Ti rotor at 32,000 x g for 10 min at 4ºC in Optima L-90K ultracentrifuge (Beckman Coulter). Fraction 3 containing total mitochondria

formed at the interphase of 40% and 26% layers was carefully removed and placed in fresh ultra-clear tubes. The tubes were topped off with isolation buffer and centrifuged in type 50.2 Ti fixed angle rotor at 16,700 x g for 10 min at 4ºC in Optima L-90K ultracentrifuge (Beckman Coulter). The supernatants were carefully removed and the loose pellets at the bottom were transferred to microcentrifuge tubes. The tubes were topped off with isolation buffer without EGTA (215mM mannitol, 75mM sucrose, 0.1% BSA, 20mM HEPES and pH is adjusted to 7.2 with KOH) and centrifuged at 13,000 x g for 10 min at 4ºC. The supernatants were carefully removed and the pellets were resuspended in 500 µL of isolation buffer without EGTA. The tubes were topped off with isolation buffer without EGTA and centrifuged at 10,000 x g for 10 min at 4ºC. The supernatants were carefully removed and the pellets were resuspended in 500 µL of isolation buffer without EGTA. The tubes were topped off with isolation buffer without EGTA and centrifuged at 10,000 x g for 5 min at 4ºC to yield a tighter pellet. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a concentration of ~ 10mg/ml. The protein concentration was determined using the BCA protein assay kit measuring absorbance at 562 nm with a BioTek Synergy HT plate reader (Winooski, Vermont).

Cryopreservation of Brain Mitochondria:

Brain Mitochondria were preserved before or after percoll purification. Dimethyl sulfoxide (DMSO) was added to the mitochondria (10% v/v). The sample was then placed in Nalgene freezing container *(catalog number: 5100-0001)* and allowed to cool to -80ºC at a uniform rate of ~ -1ºC/min. The sample can be stored at -80ºC for up to 6 months for later use.

Reanimation of Brain Mitochondria:

To reanimate the mitochondria, frozen samples were briefly thawed on ice. The sample tubes were topped off with isolation buffer with EGTA and centrifuged at 13000 x g for 10 min at 4ºC. The pellets were resuspended in 500ul isolation buffer with or without EGTA, topped off and centrifuged at 10000

x g for 10 min @ at 4ºC. The pellets were resuspended in appropriate volume. If needed, the sample can then percoll purified as described above for subsequent analysis.

Electron Microscopy:

After the last 10,000 × g centrifugation, the mitochondrial pellets were fixed in 4% glutaraldehyde overnight at 4ºC before being embedded for electron microscopy. Next, the pellets were washed overnight at 4ºC in 0.1M sodium cacodylate buffer, followed by 1 hr secondary fixation at room temperature in 1% osmium tetroxide. Then, the mitochondrial pellets were rinsed with distilled water and dehydrated for 10 min each in 70%, 85%, 95%, twice in 100% ethanol and twice in propylene oxide. The pellets were placed in a 1:1 mixture of propylene oxide and Epon/Araldite resin and infiltrated overnight on a rotator. Next, 100% Epon/Araldite resin was added and rotated for 1hr at room temperature. Finally fresh resin was prepared and degassed using a vacuum chamber. The mitochondrial pellets were added to the flat molds, filled with fresh resin, and baked overnight at 60ºC. The 90 nm sections were cut using an RMC MT-7000 ultramicrotome mounted on 150 mesh copper grids and stained with uranyl acetate and lead citrate. The sections were examined using a Zeiss 902 transmission electron microscope.

Respiration Studies:

The respiratory activity of isolated mitochondria was measured using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, England) as previously described (Brown et al. 2004; Sullivan et al. 2004). 100-120 ug/mL of isolated mitochondria were suspended in a sealed, constantly stirred, and thermostatically-controlled chamber at 37ºC in respiration buffer *(*125mM KCl, 2mM $MgCl_2$, 2.5mM KH_2PO_4, 0.1% BSA, 20mM HEPES at pH 7.2). The rate of oxygen consumption was calculated based on the slope of the response of isolated mitochondria to oxidative substrates, 5mM pyruvate and 2.5mM malate; 150µM ADP; 2µM oligomycin; 2µM FCCP; and finally 10mM succinate.

Enzyme-Linked Immunosorbent Assay (ELISA) for Cytochrome c:

Cytochrome c levels were analyzed according to the quantitative sandwich enzyme immunoassay protocol from the manufacturer (Quantikine kit; RnD Systems, Minneapolis) with slight modifications. Briefly, samples used for respiration were centrifuged at 10,000 x g for 5 min at 4ºC. The pellets were diluted to 0.01-0.03ug/ul with isolation buffer without EGTA. Samples were added to wells pre-incubated with Cytochrome c conjugate alongside known concentrations of standards. The plate was incubated in the dark for 2hr at room temperature. The contents of the wells were washed to remove unbound protein; colorimetric solution was added and incubated for 30min. Lastly, stop solution was added which turns the enzyme reaction from blue to yellow. The concentration of Cytochrome c was determined from optical density readings at 450nm (background was measured at 570nm) using Multiskan Plus (Thermo LabSystems) and expressed as ng/mg protein (% Fresh mitochondria).

Western Blotting:

Western blots for COX and VDAC were carried out as described previously (Brown et al. 2004). Briefly, samples used for respiration were centrifuged at 10,000 x g for 5 min at 4ºC. The resulting mitochondrial pellet was diluted to 1ug/ul in isolation buffer without EGTA and used for western blots. Sample buffer was added to the samples based on relative protein concentrations and boiled for 10min. Samples (5ug each) were separated by SDS-PAGE 12% Tris-bisacrylamide gels (NuPage), along with molecular weight markers (Multi-Marker, Invitrogen). Following SDS-PAGE, polypeptides were transferred electrophoretically onto 0.2 µM nitrocellulose membranes. Membranes were incubated at room temperature for 1 hour in 5% non-fat milk in 50 mM Tris-saline containing 0.05% Tween-20 at pH 7.5 (TTBS). The blots were incubated overnight in the primary antibody in TTBS at room temperature. The primary antibodies used in study included monoclonal cytochrome c oxidase subunit IV (COX IV) at 1:20,000 from Molecular Probes (Eugene, OR, USA); and polyclonal voltage-dependent anion channel (VDAC) at 1:10,000 from Affinity

Bioreagents (Golden, CO, USA). After overnight incubation in primary antibody, the membranes were rinsed three times in TTBS and incubated in secondary antibody for one hour in either HRP-conjugated goat anti-mouse IgG (1:6000) for COXIV, or HRP-conjugated goat anti-rabbit IgG (1:6000) for VDAC. The blots were rinsed thoroughly in TTBS and were developed using Pierce SuperSignal West Pico chemi-luminescent substrate and analyzed using Kodak Image-Station.

Statistical Analysis:

All results are expressed as means ± standard deviations (SD). For statistical evaluation, One-way analysis of variance (ANOVA) was performed followed by Student-Newman-Keuls post-hoc test. Significance was set at $p < 0.05$ for all analyses.

Results

Effect of Cryopreservation on Ultrastructure of Brain Mitochondria:

To determine the effects of cryopreservation on the ultra-structure of brain mitochondria, electron microscopy was performed on fresh and cryopreserved mitochondria. DMSO preserves the structure of cryopreserved brain mitochondria. Both the freshly isolated mitochondria (Figure 1A) and the cryopreserved mitochondria (Figure 1B) had intact inner and outer membranes, as well as tight cristae.

Effect of Cryopreservation on Brain Mitochondrial Bioenergetics:

To determine the effects of cryopreservation on the respiratory control ratio of brain mitochondria, oxygen consumption measurements were carried out. Freshly isolated brain mitochondria with respiratory control ratio (RCR) of greater than 5 only, obtained by dividing State 3 (presence of ADP) over State 4 respiration (absence of ADP), were used for subsequent experiments. Oxygen consumption measurements revealed that there were no significant differences in

the RCR between percoll-purified non-cryopreserved mitochondria with DMSO and crude- or purified-cryopreserved mitochondria with DMSO (Figure 2.2 and 2.3). This indicates that the electron transport system (ETS) is well coupled to the oxidative phosphorylation even upon cryopreservation. The RCR of mitochondria cryopreserved with DMSO was significantly higher from mitochondria frozen without DMSO.

To determine effects of cryopreservation on the respiratory activity of brain mitochondria, the respiratory values of each state were analyzed for fresh and cryopreserved mitochondria. We observed a significant reduction in activity in fresh mitochondria washed with DMSO compared to freshly isolated brain mitochondria in all the respiration states except States II and IV. The reduction in activity suggests a DMSO effect on the mitochondrial bioenergetics. This reduction in activity is further exacerbated in the crude and purified mitochondria cryopreserved with DMSO (Figure 2.4). However, the cryopreserved mitochondria had significantly higher activity in all states compared to the mitochondria frozen without DMSO (data not shown) indicating that DMSO, in spite of lowering the respiration by itself, does indeed preserve mitochondrial bioenergetics.

Effect of Cryopreservation on Cytochrome c levels of Brain Mitochondria:

To determine the reduction in respiratory activity upon cryopreservation, we used ELISA to analyze Cytochrome c levels of all groups. Mitochondria cryopreserved with DMSO retain significantly higher Cytochrome c levels compared to the mitochondria frozen without DMSO (Figure 2.5). However, all groups were significantly lower than the fresh non-cryopreserved brain mitochondria. Surprisingly, DMSO alone significantly reduced Cytochrome c content in the mitochondria. This loss of Cytochrome c could account for the observed reduction in the bioenergetics upon cryopreservation.

Effect of Cryopreservation on Membrane Markers of Brain Mitochondria:

Finally, to determine the possibility of mitochondrial membrane rupture resulting in reduction in cytochrome c levels, we carried out western blots for mitochondrial membrane marker proteins namely COX (inner membrane) and VDAC (outer membrane). Western blots of brain mitochondria cryopreserved with or without DMSO (Figure 2.6) demonstrated no significant differences in immunoreactivity of markers COX IV or VDAC. These data support our EM findings and taken together indicate that the mitochondrial membranes remain intact following cryopreservation.

Figure 2.1: Cryopreservation of brain mitochondria does not alter their ultra-structure. Electron microscopy pictures of fresh and cryopreserved mitochondria. The top panel (A) is a photograph of freshly isolated brain mitochondria. The bottom panel (B) is a photograph of cryopreserved mitochondria isolated from the same preparation. The small arrows indicate intact outer mitochondrial membranes whereas the large arrows indicate well-formed cristae in both the freshly isolated and cryopreserved mitochondria. (Scale bar =0.25 μm).

Figure 2.1: Cryopreservation of brain mitochondria does not alter their ultra-structure.

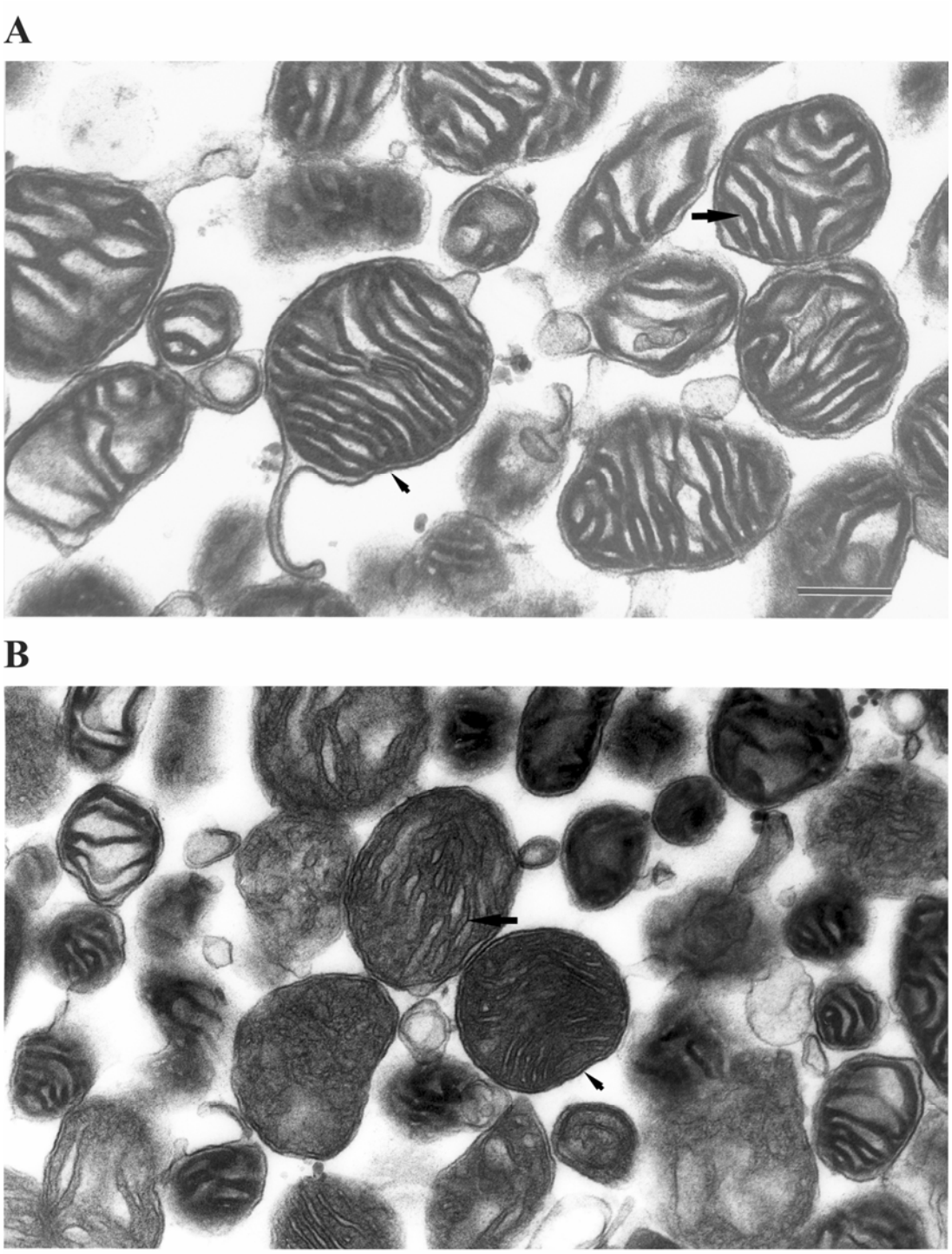


Figure 2.2: Representative Oxymetric traces of fresh and cryopreserved mitochondria obtained using a Clark-type oxygen electrode. Baseline respiration is termed as State I containing 125mM KCl respiration buffer and mitochondria (25-30ug). State II is initiated by addition of 5mM Pyruvate and 2.5mM Malate as substrates for Complex I of the electron transport chain (ETC). State III is initiated by activation of ATP Synthase (Complex V) with addition of ADP (150μM). State IV occurs when ATP Synthase is inhibited by addition of Oligomycin (2μM). FCCP (2μM) induces State V by uncoupling electron transport from oxidative phosphorylation followed by Succinate (10mM) to verify electron transport through Complex II. Freshly isolated brain mitochondria or those washed in DMSO responded very similar to crude- or purified-cryopreserved brain mitochondria whereas brain mitochondria that were frozen without cryopreservation demonstrated an obvious loss of bioenergetics.

Figure 2.2: Representative Oxymetric traces of fresh and cryopreserved mitochondria obtained using a Clark-type oxygen electrode.

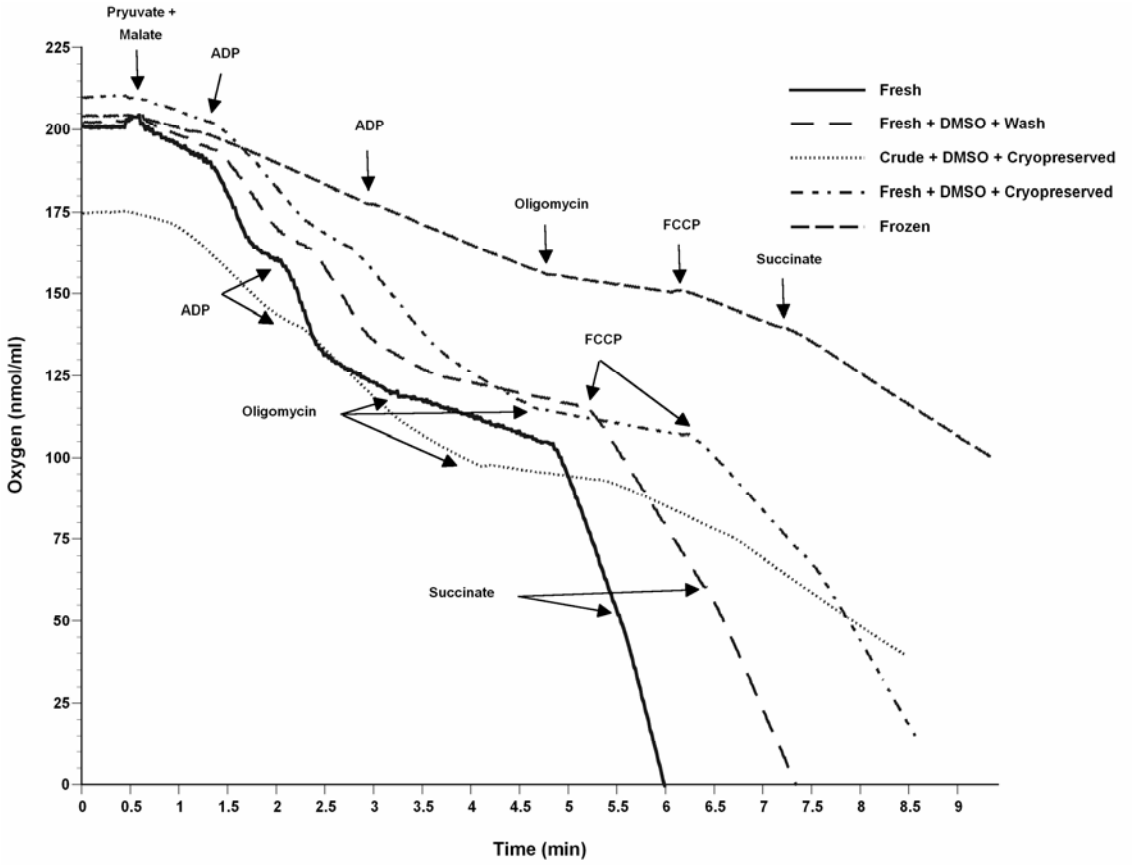


Figure 2.3: Cryopreservation of brain mitochondria preserves respiratory control ratio (RCR). Respiratory control ratios (RCR) of mitochondria, measured as State III (presence of ADP) over State IV (presence of oligomycin), demonstrate that cryopreserved brain mitochondria are well-coupled. However their RCRs were significantly lower than freshly isolated mitochondria, but no different than freshly isolated mitochondria washed with DMSO. The line represents the RCR of frozen, non-cryopreserved brain mitochondria. Data represent group means ± SD; n = 3 per group except for Fresh + DMSO + Wash (n = 2) and Frozen (n = 2). One-Way ANOVA; Student-Newman-Keuls post-hoc test; # indicates $p < 0.05$ compared to fresh mitochondria.

Measurement	DF	F – value	P – value	Power
RCR	3	8.016	0.0115	0.885

Figure 2.3: Cryopreservation of brain mitochondria preserves respiratory control ratio (RCR).

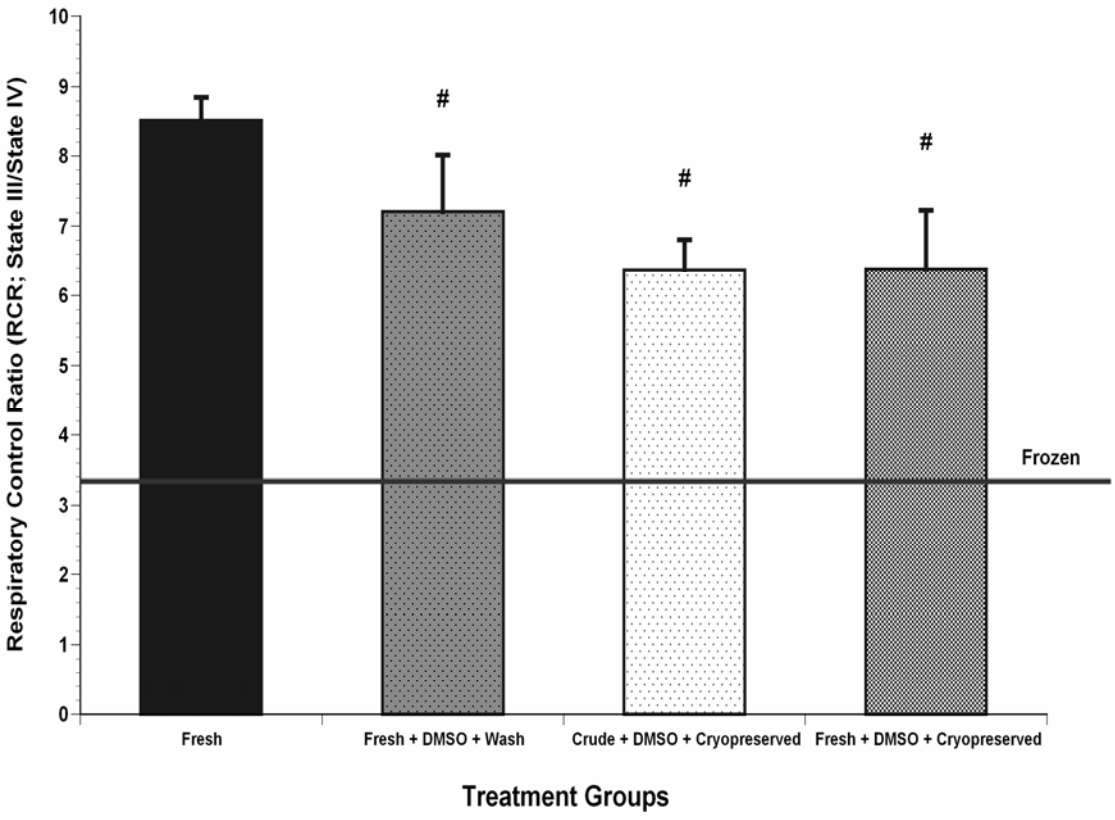


Figure 2.4: Cryopreservation of brain mitochondria preserves active bioenergetics. Although cryopreserved brain mitochondria remain well-coupled demonstrated by their RCR, they do demonstrate a significant loss of bioenergetics compared to freshly isolated mitochondria or DMSO washed mitochondria. However, this loss of electron transport system (ETS) function was also significant in DMSO washed mitochondria in States III A, III B, V and VI compared to freshly isolated mitochondria. Between the cryopreserved mitochondria, the loss in bioenergetics is markedly higher in the crude cryopreserved mitochondria compared to the purified mitochondria cryopreserved with DMSO. Data represent group means ± SD; n = 3 per group except for Fresh + DMSO + Wash (n = 2). One-Way ANOVA; Student-Newman-Keuls post-hoc test; # indicates $p < 0.05$ compared to fresh mitochondria; * indicates $p < 0.05$ compared to DMSO washed fresh mitochondria.

Measurement	DF	F – value	P – value	Power
Pyr/Mal	3	14.722	0.0021	0.992
ADP 1	3	13.658	0.0026	0.987
ADP 2	3	19.374	0.0009	0.999
Oligo	3	14.233	0.0023	0.990
FCCP	3	22.955	0.0005	1.000
Succ	3	18.626	0.0010	0.998

Figure 2.4: Cryopreservation of brain mitochondria preserves active bioenergetics.

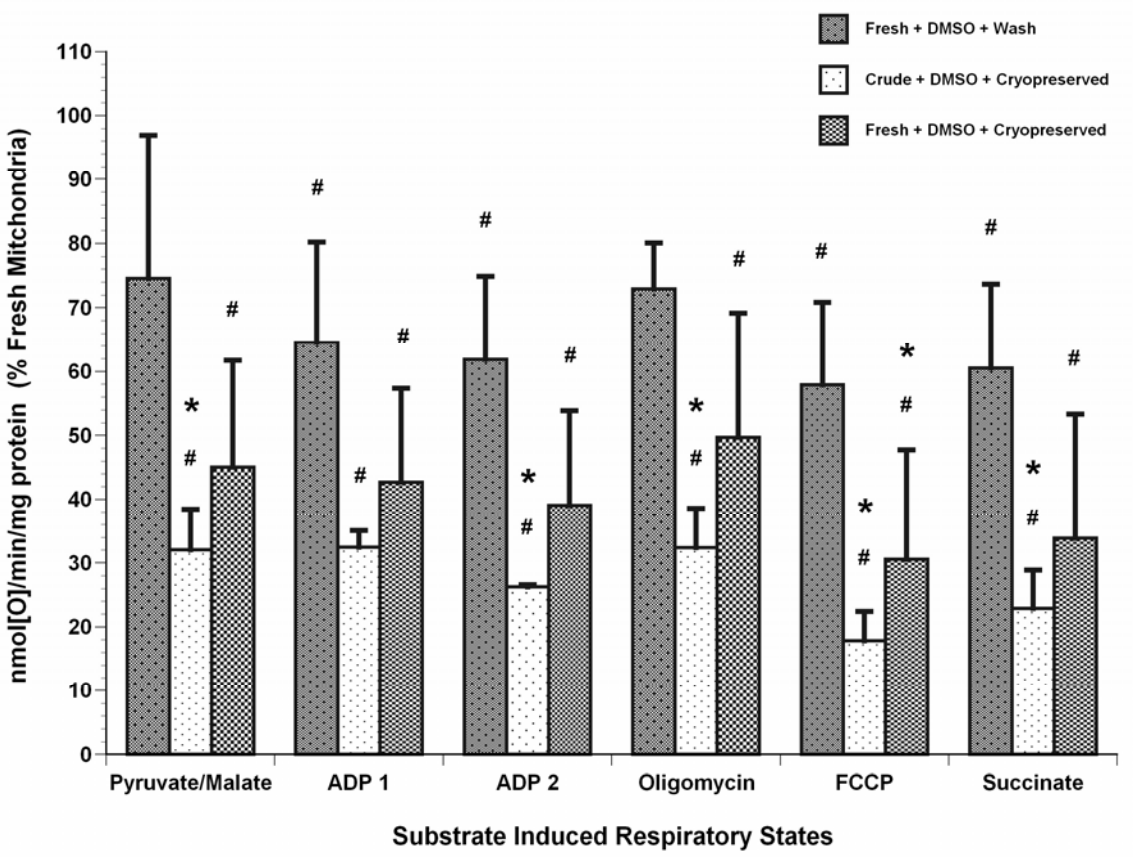


Figure 2.5: Loss of Cytochrome c in mitochondria upon cryopreservation. Mitochondrial samples were collected after respiration studies and used to assess Cytochrome c levels using ELISA. It demonstrated a significant loss of Cytochrome c in both the fresh mitochondria washed with DMSO and crude- or purified-cryopreserved mitochondria compared to freshly isolated brain mitochondria. However, mitochondria frozen without DMSO have significantly lower amounts of Cytochrome c compared to crude- or purified-cryopreserved as well as fresh mitochondria and those washed with DMSO. Data represent group means ± SD; n = 3 per group except for Fresh + DMSO + Wash (n = 2) and Frozen (n = 2). One-Way ANOVA; Student-Newman-Keuls post-hoc test; # indicates $p < 0.05$ compared to fresh mitochondria; * indicates $p < 0.05$ compared to DMSO washed fresh mitochondria; @ indicates $p < 0.05$ compared to purified-cryopreserved mitochondria.

Measurement	DF	F – value	P – value	Power
Cytochrome c	4	22.810	0.0002	1.000

Figure 2.5: Loss of Cytochrome c in mitochondria upon cryopreservation.

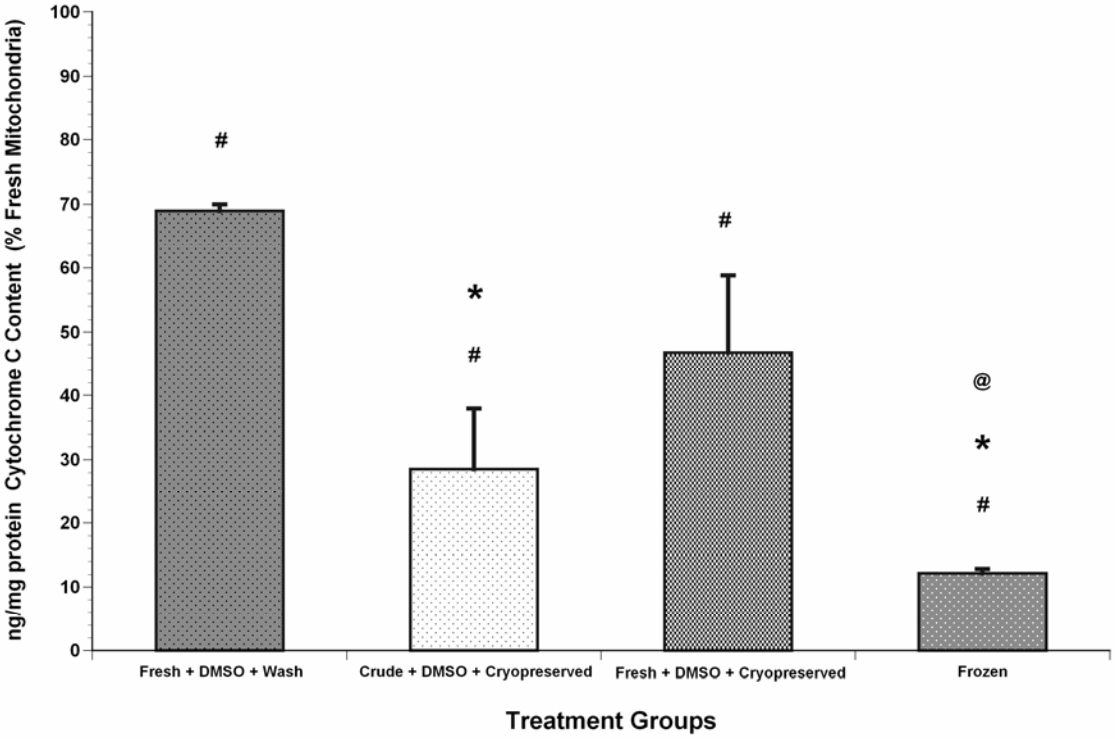


Figure 2.6: Outer and inner mitochondrial membrane marker protein levels are maintained upon cryopreservation. Aliquots of mitochondrial samples collected from respiration studies were used for Western blot analysis using a monoclonal antibody (1:20,000) against Cytochrome c oxidase (COX IV) and a polyclonal antibody against (1:10,000) against Voltage dependent anion channel (VDAC). Representative Western blots demonstrated no significant differences in COX IV (inner membrane marker) or VDAC (outer membrane marker) protein levels between fresh and cryopreserved brain mitochondria indicating that both membranes are intact.

Figure 2.6: Outer and inner mitochondrial membrane marker protein levels are maintained upon cryopreservation.

Discussion

In the present study, we show that DMSO (10% v/v) can be used for successful cryopreservation of brain mitochondria. Reanimated mitochondria exhibited preserved bioenergetics as well as intact structure. There was a reduction in Cytochrome c levels with the addition of DMSO alone which further decreased upon cryopreservation. This observation could help explain, the reduction in respiratory activity of cryopreserved mitochondria. It is not however due to rupture of mitochondrial membranes as evident both at the EM level and retention of mitochondrial membrane marker proteins upon cryopreservation.

DMSO has been initially reported to cryoprotect bull spermatozoa and human and bovine red blood cells (Lovelock and Bishop 1959) and used for cryopreservation of several other cell types. It is an oxidized thioether with a chemical formula of $(CH_3)_2SO$, thus making it water-soluble whereas its further oxidized form called as dimethyl sulfone does not exhibit any cryoprotective properties. Permeable CPAs like DMSO are highly hydrophilic and act by binding to intracellular water preventing excessive dehydration, reducing salt toxicity and thereby preventing formation of ice crystals inside the cell or in this case of isolated mitochondria. DMSO is also shown to possess hydroxyl radical scavenging capacity thus acting as an anti-oxidant (Santos et al. 2003).

One of the first reports of cryopreservation of mitochondria using DMSO appeared in the 1960's. It has been shown that DMSO and glycerol prevented decreases in respiration and oxidative phosphorylation in frozen rat liver mitochondria (Greiff and Myers 1961; Greiff et al. 1961) which was later confirmed by various groups (Lusena 1965; Lusena and Dass 1966; Dickinson et al. 1967; Fishbein and Griffin 1976). DMSO was shown to be more convenient for usage owing to its less viscosity and rapid penetration compared to glycerol. Mitochondria isolated from rat liver homogenates that had been frozen for months retained their energy-linked functions, even though DMSO by itself lowered the respiratory control ratio (Fleischer 1979; Fuller et al. 1989; De Loecker et al. 1991; Kuznetsov et al. 2003). Cryopreservation was also

successfully applied to primary rat hippocampal neurons (Mattson and Kater 1988), rat brain synaptosomes (Begley et al. 1998), rat cardiac and skeletal muscles and human biopsy samples (Kuznetsov et al. 2003). Begley et al. (1998) have shown that glucose and glutamate transport activities, mitochondrial transmembrane potential and electron transport systems maintained in cryopreserved synaptosomes were identical to freshly isolated synaptosomes and also can be successfully applied to human post-mortem brain tissues. These data indirectly provide evidence that mitochondria present in the neurons and synaptosomes were also preserved and functional.

Taking these studies into account, we decided to test whether DMSO could act as an effective CPA when added to rat cortical mitochondria. Our results support that DMSO (10% v/v) can be successfully used to cryopreserve brain mitochondria. Our lab previously demonstrated that there are no significant differences in RCR or marker proteins (COX and VDAC) in mitochondria prepared either by nitrogen-cell disruption or digitonin (Brown, Sullivan et al. 2004). In this study, we employed 0.01% digitonin to rupture the synaptoneurosomes which were further separated by discontinuous percoll gradient centrifugation to yield total (synaptic and non-synaptic pools of) mitochondria. These mitochondria were robust and well-coupled as demonstrated by their RCR. The current findings also demonstrate that freezing mitochondria without DMSO destroys their structural integrity and functional viability. Infact, freeze-thaw procedure is routinely employed to disrupt mitochondria and obtain sub-mitochondrial fractions. However, significantly higher cytochrome c levels are maintained in mitochondria cryopreserved with addition of DMSO as evident by respiration measurements. This suggests that the freeze-thaw procedures do disrupt mitochondrial membranes and that DMSO prevents most but not all damage. Infact, we did not observe any significant loss of mitochondrial membrane marker proteins (COX and VDAC) which indicates that the membranes are not ruptured and are intact, that was also evident by electron microscopy.

Clayton's group (2005) have shown that the release of cytochrome c is mediated, in part, by mitochondrial outer membrane components such as Bak and VDAC forming pores rather than by non-specific rupture or by Bax and VDAC (Brustovetsky et al. 2003). This further supports our data that cytochrome c release is due to increased leakiness of the mitochondrial outer membrane and not due to its rupture per se. One possible explanation for this observation is that cytochrome c is a low molecular weight (12kD) protein that is present in the inter-membrane space loosely bound to the mitochondrial inner membrane.

We observed a DMSO effect on the RCR and respiratory states between fresh non-cryopreserved mitochondria and the mitochondria that were incubated with 10% DMSO that was washed out immediately. This loss in activity could possibly be due to exchange of DMSO and water across the membranes and in the process causing leaking of cytochrome c as well. The loss of cytochrome c is further exacerbated upon cryopreservation. However, addition of DMSO retained significantly higher levels of cytochrome c in cryopreserved mitochondria compared to those frozen without DMSO indicating that it is indeed cryoprotective.

Kuznetsov's group (2003) have done a comparative analysis of the cryopreservative properties of glycerol and DMSO using mitochondria from permeabilized human skeletal muscles and concluded that DMSO was clearly more effective than glycerol with a better preservation obtained at concentrations between 20 to 50% with an optimal around 30%. They also observed that cryopreserved mitochondria demonstrated functional integrity, normal ATP production and energy transfer as well as structural intactness of outer mitochondrial membranes. This is in contrast to our findings that 10% DMSO causes a reduction of mitochondrial bioenergetics in part due to loss of cytochrome c.

We observed no obvious differences in the ultra-structures between the crude cryopreserved mitochondria and the percoll-purified cryopreserved mitochondria. Both the crude mitochondria cryopreserved with DMSO and the percoll-purified mitochondria cryopreserved with DMSO showed similar RCR

(>5). This suggests that the stage of cryopreservation of mitochondria (crude or purified) is not critical. Furthermore, we observed similar levels of cytochrome c and membrane marker proteins (COX and VDAC) in both the groups (Unpublished Data). This would give an opportunity to bank the crude mitochondria and reanimate and purify them at a later time for functional studies.

In summary, we report here for the first time, a practical and novel method for cryopreservation of brain mitochondria. The use of this methodology to retain the functional viability of cryopreserved mitochondria allows the banking of CNS mitochondria for comparative studies or studies to be performed in geographically isolated laboratories.

Chapter Three

Analysis of Mitochondrial Function in Post-mortem Brains of Alzheimer's (AD) and Control Patients

Introduction

Alzheimer's disease (AD) is a neurodegenerative condition marked by progressive loss of cognitive function and memory that currently affects 4 million Americans. Age is the major risk factor predisposing individuals over 65 years, in an exponential manner, to AD. Over 90-95% of AD incidences are sporadic while the rest are familial cases. Loss of synapses, deposition of extra-cellular plaques composed of β-Amyloid (Aβ) and intracellular accumulation of neurofibrillary tangles consisting mostly of tau protein polymers are hallmarks in both forms of AD pathology (Markesbery 1997; Mattson 2004; Harman 2006).

AD associated dementia is invariably accompanied by reductions in cerebral metabolic rate for glucose and cerebral blood flow, as measured *in vivo* by positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) (Farkas and Luiten 2001). They precede neuroanatomic evidence for the disease, such as increased expression of amyloid precursor protein (APP), cytoskeletal abnormalities, synaptic dysfunction and selective loss of neurons, and neuropsychological symptoms (Blass 2001).

Frontal, parietal and temporal cortices and hippocampus are some of the brain regions more vulnerable to such damage, whereas primary sensory cortex and cerebellum are not affected in AD (Scheff and Price 2003). Although altered metal ion homeostasis, aberrant protein accumulation and inflammation are shown to be involved, mechanisms implicated in aging, namely calcium dysregulation (LaFerla 2002), oxidative damage such as increased DNA oxidation, protein oxidation or nitration and lipid peroxidation (Markesbery 1997; Floyd and Hensley 2002; Perry et al. 2002; Butterfield et al. 2006; Moreira et al.

2006), are naturally extended and, in combination with impaired proteasome activity (Keller et al. 2000; Sullivan et al. 2004) and cell death by apoptosis (Mattson 2006), are shown to play an increasingly important role in the pathophysiology of AD (Mattson 2004).

Mitochondrial dysfunction is considered to play a major role in the altered metabolism observed with AD (Beal 2005; Sullivan 2005; Sullivan and Brown 2005; Parihar and Brewer 2007). This includes increased mtDNA mutations (Wallace 2005) and oxidation (Reddy and Beal 2005) and changes in mtDNA content (Hirai et al. 2001). Moreover, changes in the gene expression of ETC complexes (Manczak et al. 2004) were accompanied by decreases in activities of key mitochondrial enzymes (Gibson et al. 1998). Lastly, specific mitochondrial proteins are also shown to undergo oxidation and/or nitration (Sultana et al. 2006; Sultana et al. 2006).

Although several lines of evidence have implicated a role for mitochondrial dysfunction in AD none of the studies have directly assessed mitochondrial homeostasis. Studies that have assessed mitochondrial enzyme activities have been hindered by inconsistent results or called into question due to long (some greater than 24 hrs) postmortem intervals of the tissue (Sims 1996). The present study assessed region-specific alterations in mitochondrial homeostasis under tightly controlled conditions and a post-mortem interval (PMI) of less than 4 hours. We successfully employed the mitochondrial cryopreservation method described in Chapter 2 (Nukala et al. 2006). Synaptic and non-synaptic mitochondria were isolated from 3 regions of brain which are differentially affected in Alzheimer's disease. Mitochondria were used to measure respiration, ROS/RNS generation and oxidative damage.

Our preliminary data shows that there is a decrease in the Respiratory Control Ratios (RCR) in synaptic mitochondria isolated from AD patients compared with Control group. The respiration measured as maximum Complex I activity also decreased across all the regions in AD patients compared to Controls. While we did not notice changes in the ROS/RNS levels across the regions or between AD and Controls, we observed an increase in the levels of

protein carbonyls in all regions and across groups of AD patients. Taken together these data do suggest a role for oxidative stress and mitochondrial dysfunction in AD pathology.

Materials and Methods

Reagents:

Mannitol, sucrose, bovine serum albumin (BSA), ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), hydroxyethyl piperazine-1-ethanesulfonic acid potassium salt (HEPES), potassium phosphate monobasic anhydrous (KH_2PO_4), magnesium chloride ($MgCl_2$), potassium chloride (KCl), malate, pyruvate, adenosine 5'-diphosphate (ADP), digitonin, succinate and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oligomycin A and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) were purchased from Biomol (Plymouth Meeting, PA). Dihydrodichloro fluorescein diacetate (H_2DCF-DA) was obtained from Molecular Probes (Eugene, OR) and bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL).

Brain Autopsy Samples from AD and Control Patients:

The graduate student was on-call to collect brain tissue samples from Alzheimer's patients (n=4; PMI ≈ 3.125hrs) and age-matched controls (n=2; PMI ≈ 2.5hrs) enrolled and longitudinally followed by the Sanders-Brown Center on Aging Alzheimer's Disease Research Center (ADRC). Dr. William Markesbery, Director of Neuropathology Core, led the Rapid Autopsy Team to perform short postmortem interval (PMI) autopsies using a standardized protocol on longitudinally followed patients with AD and other age-related dementing disorders and cognitively normal aged, longitudinally followed, control subjects. Dr. David Wekstein, Director of Education and Information Core, chief contact person along with Dr. James Geddes and Dr. Steve Scheff were available at the autopsies.

Table 3.1: Brain Autopsy Samples from AD and Control Patients

Case #	Group	Sex (M/F)	Age (yrs)	PMI (hrs)
1094	Control	Male	70	3.0
1097	Control	Female	89	1.75
1086	AD	Female	90	2.75
1090	AD	Female	84	1.75
1096	AD	Male	84	4.5
1101	AD	Female	86	3.25

Isolation and Purification of Brain Mitochondria:

This protocol contains modifications of previously described procedures (Brown et al. 2004; Brown et al. 2006; Nukala et al. 2006). All procedures were performed on ice throughout the protocol. A 1-2 cm punch of tissue from frontal (F) cortex, middle temporal (MT) gyrus and sensory (S) cortex were collected at autopsy, minced and placed into a large manual glass dounce homogenizer containing 15 ml of ice-cold isolation buffer with EGTA and protease-inhibitor cocktail (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 1 mM EGTA, 20 mM HEPES, pH adjusted to 7.2 with KOH). The tissue was homogenized and mitochondria were isolated by differential centrifugation. The homogenate is transferred to 50ml conical tubes (total volume of 35-40mls) and centrifuged at 1300 x g for 5 min @ 4ºC using Eppendorf 5810-R table-top centrifuge. The resulting supernatant transferred to fresh tubes to a total volume of 35-40mls. The pellet is resuspended in the buffer again and centrifuged at 1300 x g for 5 min @ 4ºC as above and the resulting supernatant transferred is to fresh tubes. The supernatants from these two spins are centrifuged at 13,000 x g for 10 min @ 4ºC. The supernatant was discarded and the resulting pellet was resuspended in 1-2mls of isolation buffer with EGTA. At this point, 10% (v/v) DMSO was added to the mitochondria, and were cryopreserved as described in chapter 1 (Nukala et al. 2006).

Upon reanimation, the samples were further purified by percoll density gradient centrifugation. Stocks of 40%, 26% and 24% percoll were prepared fresh in isolation buffer with EGTA. 3.5 ml of 26% stock was carefully layered over 3.5 ml of 40% in 13 ml ultra-clear tubes. Equal volume of 24% percoll was added to the sample to get a 12% final concentration and loaded gently on top of the 26% layer. The sample was centrifuged in fixed angle type 50.2 Ti rotor at 32,000 x g for 10 min at 4ºC in Optima L-90K ultracentrifuge (Beckman Coulter). Non-synaptic mitochondria are collected as Fraction 3 formed at the interphase of 40% and 26% layers and was carefully removed and placed in fresh ultra-clear tubes. The synaptoneurosomes as Fraction 2 at the interphase of 26% and 24% are carefully removed. The synaptoneurosomal mitochondria are then released by use of a nitrogen cell bomb to avoid the conflicts of detergent based methods of synaptoneurosome rupture. The tubes were topped off with isolation buffer with EGTA and centrifuged in type 50.2 Ti fixed angle rotor at 16,700 x g for 10 min at 4ºC in Optima L-90K ultracentrifuge (Beckman Coulter). The supernatants were carefully removed and the loose pellets at the bottom were transferred to microcentrifuge tubes. The tubes were topped off with isolation buffer without EGTA (215mM mannitol, 75mM sucrose, 0.1% BSA, 20mM HEPES and pH is adjusted to 7.2 with KOH) and centrifuged at 10,000 x g for 10 min at 4ºC. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a concentration of ~ 10mg/ml. This method yields synaptic and non-synaptic mitochondria from each region of interest. The protein concentration was determined using the BCA protein assay kit measuring absorbance at 562 nm with a BioTek Synergy HT plate reader (Winooski, Vermont).

Respiration Studies:

The respiratory activity of isolated mitochondria was measured using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, England) as previously described (Brown et al. 2004; Nukala et al. 2006). Mitochondria were suspended in a sealed, constantly stirred, and thermostatically-controlled chamber at 37ºC in respiration buffer *(*215 mM Mannitol, 75 mM Sucrose, 2mM

$MgCl_2$, 2.5mM KH_2PO_4, 0.1% BSA, 20mM HEPES at pH 7.2). The rate of oxygen consumption was calculated based on the slope of the response of isolated mitochondria to oxidative substrates, 5mM pyruvate and 2.5mM malate; 150μM ADP; 2μM oligomycin; 2μM FCCP; and finally 10mM succinate.

Fluorescent Spectrometry to Measure Mitochondrial ROS/RNS Generation:

Mitochondrial ROS/RNS production was measured using 10μM dihydrodichloro fluorescein diacetate (H_2DCF-DA) obtained from Molecular Probes (Eugene, OR) with Ex: 485nm and Em: 530nm in presence of 25U/ml HRP in the Biotek Synergy HT plate reader as previously described (Brown et al. 2006). Isolated mitochondria (100ug) were added to 100 μl of respiration buffer (see above) with 5 mM pyruvate and 2.5 mM malate as oxidative substrates and incubated at 37ºC. ROS production was calculated as the rate of dichloro fluorescein (DCF) fluorescence over 15min of incubation, expressed in arbitrary fluorescence units.

Western Blotting:

For determination of protein carbonyls, the OxyBlot Protein Oxidation Detection Kit (Chemicon) was used according to manufacturer's specifications. Each sample (20ug) was loaded onto SDS-PAGE 12% gels and ran at 100volts for about 1.5hrs at room temperature following which they were electrophoretically transferred onto nitrocellulose membranes over 1hr at 4ºC. The membranes were blocked in 5% non-fat milk in TTBS for 1hr. The membranes were then incubated with polyclonal antibody against dinitrophenyl hydrazone (DNP) at 1:1000 overnight with agitation at 4ºC. The membranes were washed with TTBS three times and incubated with the secondary Goat-α-Rabbit antibody for 2hrs. The membranes were washed three times with TTBS and incubated with the ECL reagent (GE Healthcare) and the film was developed in a dark room.

Statistical Analysis:

All results are expressed as means ± SD. For statistical evaluation, Student's t-test was performed. Significance was set at $p < 0.05$ for all analyses.

Results:

Mitochondrial RCR in AD and Control Patients:

To assess the extent of mitochondrial dysfunction in Alzheimer's disease (AD), we carried out respiration studies directly in synaptic mitochondria isolated from various regions of post-mortem brains from AD and control patients. The respiratory control ratio, described as State III/State IV and expressed as average of all the regions, is lower (~ 60%) in brain samples from AD patients (RCR = 2.69 ± 0.68) compared to Control group (RCR = 6.4 ± 2.97), approaching significance ($p = 0.0551$) (Figure 3.1).

Mitochondrial Maximum Complex I Activity in AD and Control Patients:

We analyzed the maximum Complex I activity in synaptic mitochondria (defined as the respiration induced by FCCP in presence of Complex I substrates) and observed that it is lower in the entire three – mid-temporal (MT), frontal (F) and sensory (S) regions of AD patients compared to control patients. The differences were most prominent, approaching significance ($p = 0.0669$), between the frontal region (~ 75%) of AD and Control patients. The activity was lower by ~ 50% in MT and ~ 70% in S regions of AD compared to those of Control patients but not significant.

Mitochondrial ROS/RNS Generation in AD and Control Patients:

We did not observe any changes in ROS/RNS levels in synaptic mitochondria from any of the regions between AD and Control patients. However, within AD, middle-temporal (MT) seems to have a higher ROS/RNS generation compared to frontal (F) and sensory (S) regions.

Mitochondrial Oxidative Damage in AD and Control Patients:

Finally, we measured levels of protein carbonyls, using western blots, as a measure of the extent of protein oxidation in both synaptic and non-synaptic mitochondria. The synaptic mitochondria show increased protein carbonyls in AD patients compared to synaptic mitochondria of Control group. Further, the non-synaptic mitochondria of AD patients tend to show increased protein carbonyls across all regions compared to non-synaptic mitochondria of Control group. Lastly, we also observed increased protein carbonyls in non-synaptic mitochondria compared to synaptic mitochondria across all regions within AD and Controls. Overall, there is higher oxidative damage in mitochondria from AD patients compared to Control group.

Figure 3.1: Mitochondrial RCR decreased in AD patients. Mitochondrial respiration was carried out at 37ºC by incubating mitochondria in respiration buffer and oxygen consumption of mitochondria was measured in response to addition of various substrates and inhibitors. The respiratory control ratio (RCR), measured as State III (presence of ADP) over State IV (presence of Oligomycin) respiration, is lower, approaching significance ($p = 0.0551$) , in mitochondria from AD patients compared to Control group. Data represent group means ± SD; $n = 4$ per AD group and $n = 2$ per Control group; Student's t-test.

Measurement	DF	t – value	P – value
RCR	4	- 2.682	0.0551

Figure 3.1: Mitochondrial RCR decreases in AD patients.

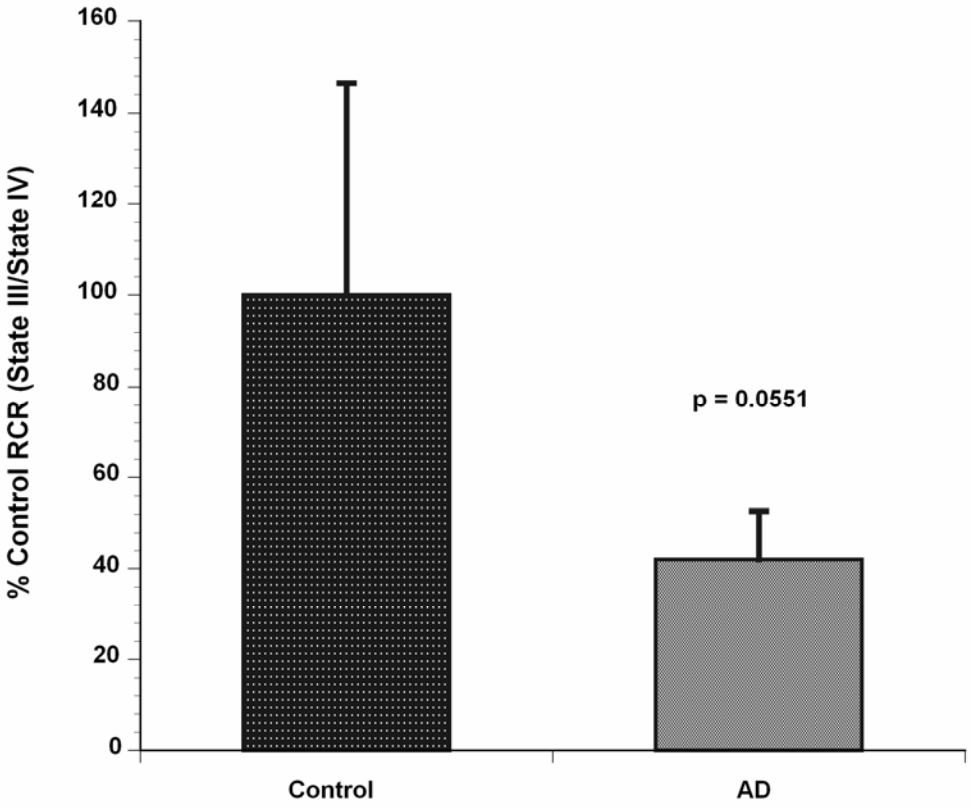


Figure 3.2: Mitochondrial maximum Complex I activity decreased in AD patients. The maximum Complex I activity, defined as the FCCP induced respiration in presence of Complex I substrates (pyruvate and malate) is lower in middle-temporal (MTs), frontal (Fs) and sensory (Ss) regions of synaptic mitochondria from AD compared to Control group. However, the differences are not significant in any of the regions assessed. Data represent group means ± SD; n = 4 per AD group and n = 2 per Control group; Student's t-test.

Measurement	DF	t – value	P – value
Mid-temporal (MT)	4	- 2.044	0.1105
Frontal (F)	4	- 2.499	0.0669
Sensory (S)	3	- 1.666	0.1944

Figure 3.2: Mitochondrial maximum Complex I activity decreased in AD patients.

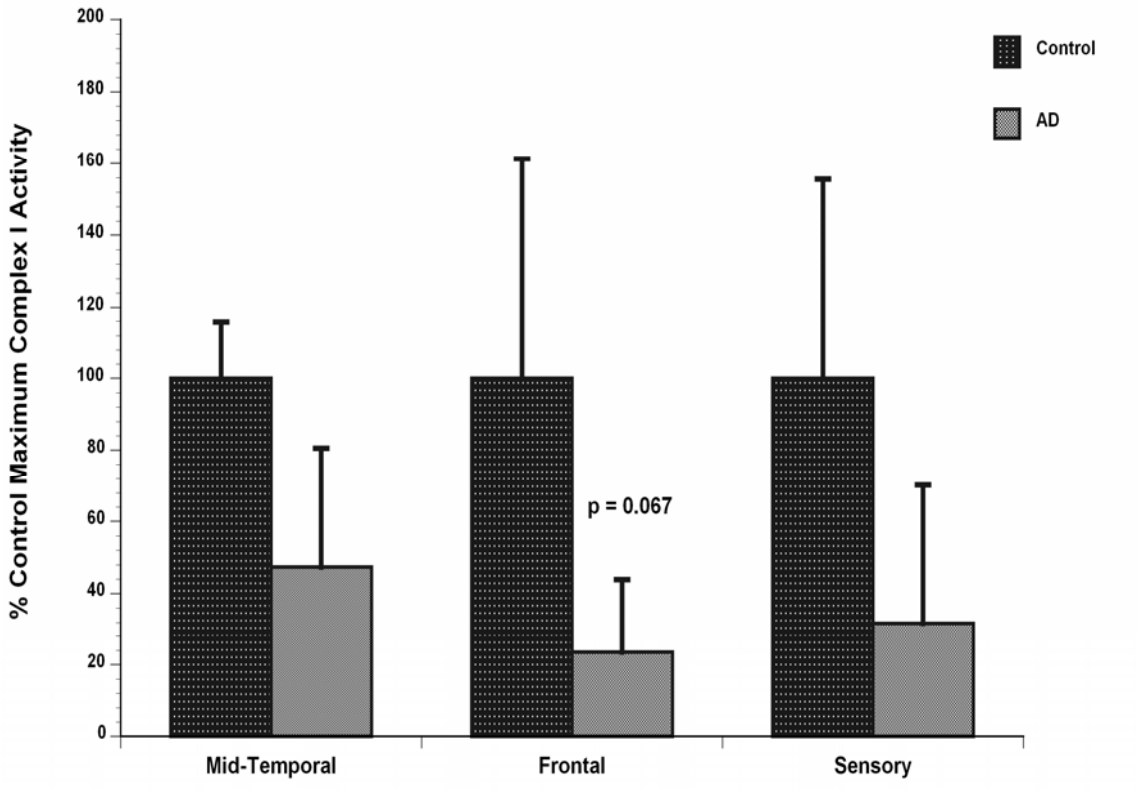


Figure 3.3: No changes in mitochondrial ROS/RNS levels in AD and Control patients. Mitochondria were incubated in respiration buffer at 37ºC containing 5 mM pyruvate and 2.5 mM malate as oxidative substrates and the reaction was carried out for 15min. There were no changes in DCF fluorescence (Ex: 485nm; Em: 530nm), as a measure of free radical production, in middle-temporal (MTs), frontal (Fs) and sensory (Ss) regions of synaptic mitochondria from AD compared to Control group. Data represent group means ± SD; n = 4 per AD group and n = 2 per Control group; Student's t-test.

Measurement	DF	t – value	P – value
Mid-temporal (MT)	4	0.635	0.5602
Frontal (F)	3	-	-
Sensory (S)	3	- 1.173	0.3255

Figure 3.3: No changes in mitochondrial ROS/RNS levels in AD and Control patients.

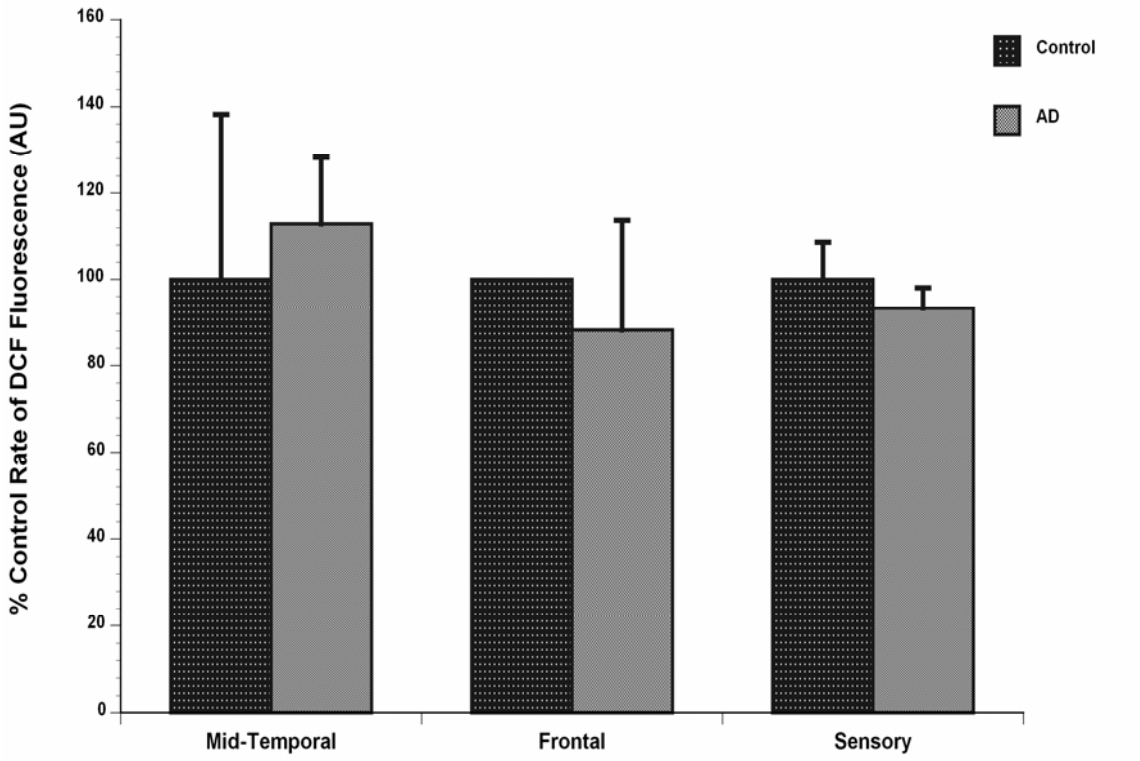
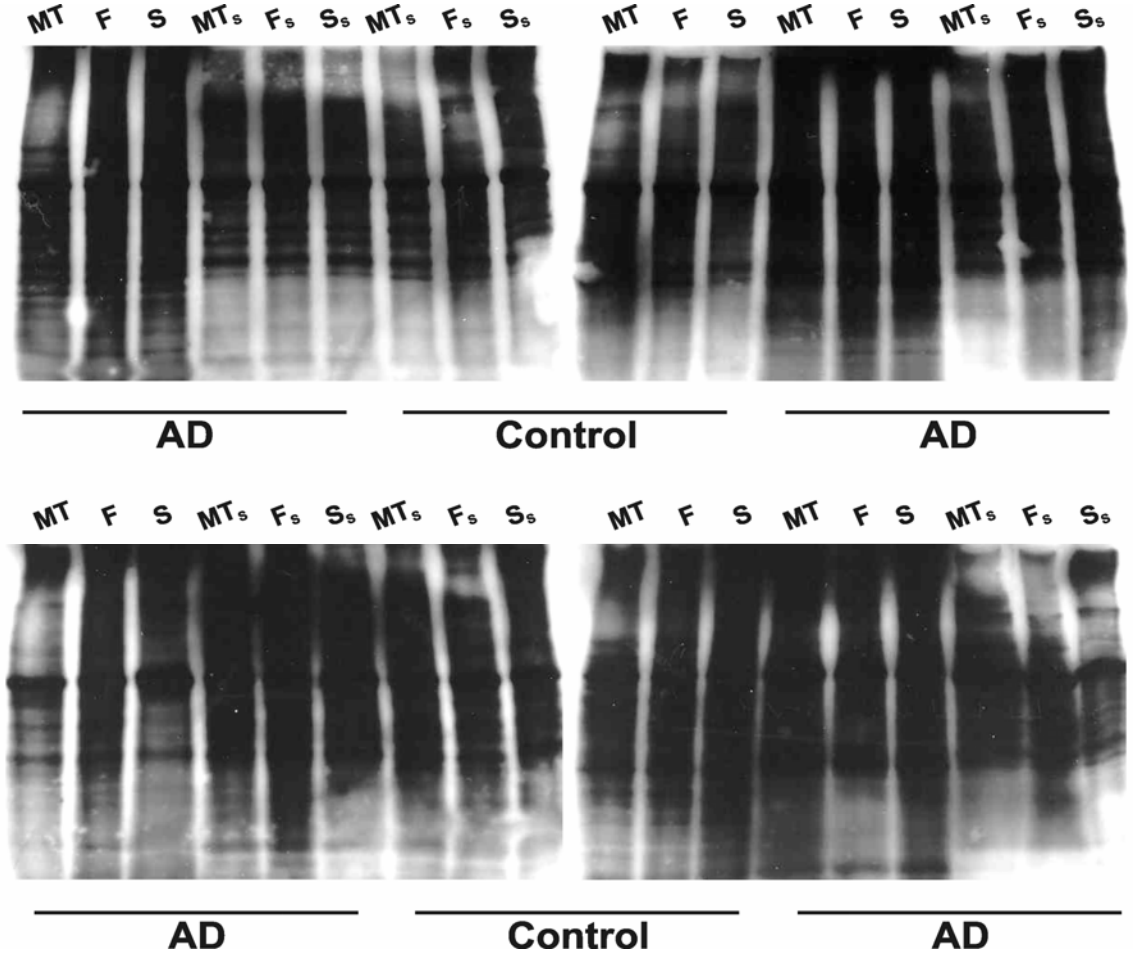


Figure 3.4: Mitochondrial oxidative damage is higher in AD patients. A OxyBlot Protein Oxidation Detection Kit was used in combination with western blots. The levels of protein carbonyls were probed using an antibody against DNP moieties (1:1000) of oxidized proteins. They were found to be higher in both synaptic and non-synaptic mitochondria across all regions of AD patients compared to Controls. Moreover, the protein carbonyls were higher in non-synaptic [Mid-Temporal (MT), Frontal (F) and Sensory (S)] mitochondria compared to synaptic [Mid-Temporal (MTs), Frontal (Fs) and Sensory (Ss)] mitochondria across all regions within AD and Controls. n = 4 per AD group and n = 2 per Control group.

Figure 3.4: Mitochondrial oxidative damage is higher in AD patients.



Discussion:

In the present study, we carried out experiments directly on mitochondria isolated from various regions of post-mortem brains and show mitochondrial dysfunction in AD patients compared to age-matched Control group. We were able to successfully use cryopreserved mitochondria as described in Chapter 1. We observed lower respiratory control ratio (RCR) as well as decreases in the maximum Complex I activity in synaptic mitochondria from middle-temporal (MT), frontal (F) and sensory (S) regions of AD brains. While we did not observe any changes in mitochondrial ROS/RNS levels in any of the groups, we were able to detect increased oxidative damage in AD mitochondria compared to Controls.

Most of the studies involving mitochondria in AD were performed on mitochondria isolated from biopsy samples or post-mortem brains (Sims 1996). This resulted in data where no changes were observed with biopsy tissue or data with highly variable results due to the differences in the post-mortem intervals (PMI). We had the distinct advantage of having the graduate student available on an on-call basis to obtain brain samples from the University of Kentucky Alzheimer's Disease Research Center (ADRC) with a post-mortem interval of under 4hours. This eliminates some of the variability observed and helps achieve more consistent data (Hynd et al. 2003).

Mitochondria can be cryopreserved using dimethyl sulfoxide (DMSO) and can be retrieved with preserved structure and function (Nukala et al. 2006). We employed this technique to cryopreserve mitochondria obtained from post-mortem samples from AD and Control brains. We were able to rapidly isolate mitochondria after collecting the brain samples within a couple of hours from the time of death. This is the first such study that enables laboratories to bank valuable post-mortem samples as well as conduct functional studies directly on mitochondria from such tissues.

Mitochondrial dysfunction and the resulting altered metabolism is implicated in both aging and Alzheimer's disease (Beal 2005; Sullivan and Brown 2005; Parihar and Brewer 2007). There are increased mitochondrial DNA

(mtDNA) mutations in AD brains, in the form of point mutations (Lin et al. 2002) or rearrangements preferentially in the transcription and replication regulatory elements of mtDNA, with the inevitable consequence of defective ETC complexes (Wallace 2005). This is also evident from altered mRNA expression in NADH dehydrogenase and ATP Synthase, down-regulation of expression of Complex I and up-regulation of Complex III and IV subunits (Manczak et al. 2004). Moreover, mtDNA had higher oxidized bases in the frontal, parietal and temporal lobes of AD brains and was 10-fold higher than nuclear DNA (nDNA) in the same tissues (Hirai et al. 2001; Wang et al. 2005). Lastly, significant decreases of mitochondria in selective neuronal cell populations were observed with AD. This was accompanied by increased mtDNA and protein content mainly in autophagic vacuoles and the cytoplasm (Hirai et al. 2001).

In addition, reductions in key enzymatic activities were observed in mitochondria of AD patients (Gibson et al. 1998). Specifically, pyruvate dehydrogenase complex (PDHC), α-ketoglutarate dehydrogenase complex (KGDHC) and cytochrome c oxidase (COX) show decreased activity in the regions affected in AD. PDHC catalyzes the conversion of pyruvate from glycolysis to acetyl-CoA that enters the Kreb's cycle. Interestingly, pyruvate dehydrogenase (PDHC) and α-ketoglutarate dehydrogenase complex (KGDHC) are two of the three mitochondrial dehydrogenases that are activated by calcium (Gunter et al. 2004). KGDHC converts α-ketoglutarate to succinyl-CoA within Kreb's cycle. Alternately, KGDHC can also convert α-ketoglutarate to glutamic acid involved with glutamate metabolism. COX, on the other hand, is the last electron acceptor and donator in the ETC where, oxygen is reduced to water. Inhibition or decrease in activity of any the above enzymes would limit the substrate availability to the ETC and therefore reduce ATP generation (Blass 2000).

Based on above observations, we assessed mitochondrial respiration directly by measuring mitochondrial oxygen consumption in synaptic mitochondria. We observed a decrease in the respiratory control ratio (RCR) in AD patients of about ~ 45% of the age-matched control group (Figure 3.1). This

supports a previous report by Sims. *et al*., where they have observed a similar decrease in RCR (~ 60% of controls) in brain homogenates from regions affected in AD patients (Sims et al. 1987). In the same study, they did not observe changes in the maximal respiratory rate (in presence of an uncoupling agent) between AD and control subjects. In contrast, our data shows a marked decrease in the maximal respiration activity (induced by Complex I substrate) in presence of FCCP (Figure 3.2) in mitochondria of AD subjects. The discrepancy could possibly be due to the source of the mitochondria i.e. brain homogenates in the former versus purified mitochondria in the present study (Sims 1996). Moreover, the decreases were observed in all the three regions assessed, with most pronounced decreases in the frontal cortex.

β-Amyloid (Aβ) is one of the mechanisms shown to increase oxidative stress in the mitochondria (Canevari et al. 2004; Caspersen et al. 2005; Reddy 2006). In fact, both Aβ and its precursor protein APP are shown to be capable of entering mitochondria. Aβ can interact with pyruvate dehydrogenase complex (PDHC) and also inhibit Complexes III and IV of the ETC thus inhibiting mitochondrial respiration. It is also shown to lower mitochondrial membrane potential and ATP levels and increase free radicals. These free radicals in turn can activate secretases that can cleave APP into Aβ. Furthermore, Aβ is also shown to cause release of cytochrome c and caspase activation in functional mitochondria. Another source of mitochondrial superoxide, besides the ETC, is the KGDHC (Markesbery 1997; Perry et al. 2002; Starkov et al. 2004; Moreira et al. 2006) that also interacts with Aβ. All the above reasons make mitochondria a prime breeding ground for oxidative stress and damage leading to their dysfunction as seen with AD.

We therefore carried out experiments to measure the free radical generation in synaptic mitochondria from AD and control subjects. We did not detect any differences in ROS/RNS levels in any of the regions from either group (Figure 3.3). This could be due to the fact that these studies were carried out in post-mortem tissues and might have missed out on the window to detect such changes. Moreover, such tissues might have altered antioxidant enzyme

activities or increased scavenging activity for which there are no consistent reports. Alternately, Aβ-induced free radical generation may not be a major pathway for the observed oxidative stress. Therefore, a more reliable indicator would be to measure the markers of oxidative damage as seen with numerous published studies (Markesbery 1997; Perry et al. 2002; Moreira et al. 2006). We measured changes in protein carbonyls as a marker for protein oxidation, although it detects protein-lipid peroxidation adducts as well (Sultana et al. 2006). They were higher in both synaptic and non-synaptic mitochondria across all regions of AD patients compared to Controls. Moreover, the protein carbonyls were higher in non-synaptic mitochondria compared to synaptic mitochondria across all regions within AD and Controls. These differences could be due to the variable properties of neuronal (synaptic) and non-neuronal mitochondria (Park et al. 2001; Brown et al. 2004; Vergun and Reynolds 2005; Brown et al. 2006; Chang and Reynolds 2006).

In fact our lab (Brown et al. 2004; Brown et al. 2006) and others (Park et al. 2001; Vergun and Reynolds 2005) have shown that the different sets of mitochondria respond differently in undergoing mitochondrial membrane permeability transition (MPT) to similar calcium loads. In addition, Butterfield's group, using redox proteomics, has shown that key mitochondrial proteins such as VDAC and ATP Synthase α-chain undergo protein oxidation as well as nitration (Sultana et al. 2006; Sultana et al. 2006). Lastly, It is well known that cytochrome c oxidase (COX) is reversibly inhibited by nitric oxide at low concentrations and irreversibly at higher concentrations through peroxynitrite (Brown and Borutaite 2002; Moncada and Bolanos 2006). This is possibly induced through a calcium-activated nitric oxide synthase (mtNOS) localized to the mitochondria (Giulivi et al. 2006; Cooper and Giulivi 2007). Based on previous literature on calcium dysregulation, increases in free radicals and our own data showing decreases in mitochondrial respiration and higher oxidative damage, the existence of and role for a mitochondrial nitric oxide synthase (mtNOS) requires further elucidation and therefore explored in the next chapter.

Chapter Four

Relationship Between Calcium and Nitric Oxide Synthase in Brain Mitochondrial Dysfunction

Introduction

Various reports can be found in recent literature on the existence of nitric oxide synthase enzyme (mtNOS) localized to mitochondria (Bates et al. 1995; Tatoyan and Giulivi 1998; Kanai et al. 2001; Lacza et al. 2001; Elfering et al. 2002; Riobo et al. 2002; Haynes et al. 2004). Evidence in rat liver mitochondria indicates that mtNOS exhibited Ca^{2+}–dependent increase in activity, which was blocked by NOS inhibitors and might be involved in mitochondrial O_2 consumption and membrane potential (Ghafourifar and Richter 1997). It has been suggested that NOS present within the mitochondria could be the source of NO• observed (Giulivi et al. 1998). Various groups have clearly established that NO• inhibits cytochrome c oxidase in intact mitochondria reversibly by competing with oxygen (Radi et al. 2002; Persichini et al. 2005). *In vitro* studies indicated that peroxynitrite ($ONOO^-$) is involved in release of Ca^{2+} thereby preserving membrane potential (Bringold et al. 2000).

It was shown that uptake of Ca^{2+} by mitochondria stimulates mtNOS causing release of cytochrome c involving $ONOO^-$, suggesting that Ca^{2+} – induced apoptosis is partly mediated via mtNOS (Ghafourifar et al. 1999). There is ample evidence showing that NO• and its derivatives such as $ONOO^-$ are involved in inhibition of respiration at the level of ETC as well as alter other mitochondrial proteins (Brown and Borutaite 2002; Radi et al. 2002; Elfering et al. 2004; Traaseth et al. 2004). Peroxynitrite ($ONOO^-$) is also capable of causing lipid peroxidation (Violi et al. 1999) as well as DNA modification (Aust and Eveleigh 1999). On the other hand, the type of NOS isoform in the mitochondria, the availability of substrates and cofactors for the enzyme, Ca^{2+} dependency and

it putative physiological function and pathological effects have been called into question (French et al. 2001; Lacza et al. 2003; Brookes 2004; Gao et al. 2004; Ghafourifar and Cadenas 2005; Lacza et al. 2006).

Several lines of evidence suggest that perturbations in intracellular Ca^{2+} homeostasis are involved in mitochondrial mediated apoptotic pathway. However, the sequelae of events leading from mitochondrial Ca^{2+} overload to RNS mediated damage in the mitochondria are still unclear. It can be postulated that inactivation of key respiratory and anti-oxidant enzymes, lipid peroxidation and DNA modifications by NO• and $ONOO^-$ could cause increased oxidative stress, ATP depletion due to mitochondrial dysfunction and subsequent cell death. The sources of NO• and its derivatives responsible for the observed effects are not yet understood completely. In light of these findings and gaps therein, we measured the effects of calcium on mitochondrial respiration, free radical generation and oxidative damage markers, in addition to NOS activity and NOS protein in purified mitochondria.

Our results confirm the presence of an active NOS enzyme inside the mitochondria that is not due to cytosolic contamination. We also show that calcium inhibits mitochondrial respiration in a dose- and time-dependent manner as well as increases ROS/RNS levels, although either of those two parameters was not reversed in presence of NOS inhibitors. There were detectable but not significant changes in levels of oxidative damage markers with exposure to patho-physiological doses of calcium. Taken together, our data suggests that although calcium induces mitochondrial dysfunction the mechanism may not entirely be via the mtNOS pathway.

Materials & Methods

Reagents:

Mannitol, sucrose, bovine serum albumin (BSA), ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), hydroxyethyl piperazine-1-

ethanesulfonic acid potassium salt (HEPES), potassium phosphate monobasic anhydrous (KH_2PO_4), magnesium chloride ($MgCl_2$), malate, pyruvate, adenosine 5'-diphosphate (ADP), succinate, calcium chloride ($CaCl_2$), L-Arginine (L-Arg), potassium chloride (KCl), proteinase-K and horseradish peroxidase (HRP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dihydro dichlorofluorescin diacetate (H_2DCF-DA), tetramethylrhodamine ethyl ester (TMRE) were purchased from Molecular Probes (Eugene, OR). Oligomycin A, rotenone and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) were purchased from Biomol (Plymouth Meeting, PA). N^G-Nitro-L-arginine (L-NNA) and N^G-Nitro-L-arginine methyl ester, hydrochloride (L-NAME) were purchased from Calbiochem (San Diego, CA). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL).

Isolation and Purification of Brain Mitochondria:

All experimental protocols involving animals were approved by the University of Kentucky Animal Use and Care Committee. This protocol contains modifications of previously described procedures (Brown et al. 2004; Nukala et al. 2006). All procedures were performed on ice throughout the protocol. Male Sprague-Dawley rats (~250-300g) were decapitated and the brains were rapidly removed. The cortices were dissected out and placed in an all-glass dounce homogenizer containing five times the volume of isolation buffer with 1 mM EGTA (215 mM mannitol, 75 mM sucrose, 0.1 % BSA, 20 mM HEPES, 1 mM EGTA and pH is adjusted to 7.2 with KOH). The tissue was homogenized and mitochondria were isolated by differential centrifugation. Briefly, the homogenate was centrifuged at 1300 x g for 3 minutes twice in an eppendorf microcentrifuge at 4ºC and the supernatant was transferred to new tubes. The resulting supernatant was transferred to new microcentrifuge tubes and topped off with isolation buffer with EGTA and centrifuged at 13,000 x g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 500 µL of isolation buffer with EGTA. To obtain maximal yield of total (synaptic and non-synaptic) mitochondria, synaptosomal membranes were disrupted by nitrogen cell

disrupter incubated for 10 min at 1200psi. The sample was further purified by percoll density gradient centrifugation. Stocks of 40%, 24% and 30% percoll were prepared fresh in isolation buffer with EGTA. 3.5 ml of 24% stock was carefully layered over 3.5 ml of 40% in 13 ml ultraclear tubes. Equal volume of 30% percoll was added to the sample to get a 15% final concentration and loaded gently on top of the 24% layer. The sample was centrifuged in fixed angle type SE-12 rotor at 30,400 x g for 10 min at 4ºC in RC 5C Plus (Sorvall) high-speed centrifuge. Fraction 3 containing total mitochondria formed at the interphase of 40% and 24% layers was carefully removed and placed in fresh ultraclear tubes. The tubes were topped off with isolation buffer without EGTA (215 mM mannitol, 75 mM sucrose, 0.1 % BSA, 20 mM HEPES and pH is adjusted to 7.2 with KOH) and centrifuged in fixed angle SE-12 rotor at 16,700 x g for 15 min at 4ºC in RC 5C Plus (Sorvall) high-speed centrifuge. The supernatants are carefully removed. The tubes were topped off with isolation buffer without EGTA and centrifuged in fixed angle SE-12 rotor at 13,000 x g for 10 min at 4ºC in RC 5C Plus (Sorvall) high-speed centrifuge. The supernatants were carefully removed and the loose pellets at the bottom were transferred to microcentrifuge tubes. The tubes were topped off with isolation buffer without EGTA and centrifuged at 13,000 x g for 10 min at 4ºC. The supernatants were carefully removed and the pellets were resuspended in 500 µL of isolation buffer without EGTA. The tubes were topped off with isolation buffer without EGTA and centrifuged at 10,000 x g for 5 min at 4ºC to yield a tighter pellet. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a concentration of ~ 10mg/ml. The protein concentration was determined using the BCA protein assay kit measuring absorbance at 562 nm with a BioTek Synergy HT plate reader (Winooski, Vermont).

Electron Microscopy:

After the last 10,000 × g centrifugation, the mitochondrial pellets were fixed in 4% glutaraldehyde overnight at 4ºC before being embedded for electron microscopy. Next, the pellets were washed overnight at 4ºC in 0.1M sodium

cacodylate buffer, followed by 1 hr secondary fixation at room temperature in 1% osmium tetroxide. Then, the mitochondrial pellets were rinsed with distilled water and dehydrated for 10 min each in 70%, 85%, 95%, twice in 100% ethanol and twice in propylene oxide. The pellets were placed in a 1:1 mixture of propylene oxide and Epon/Araldite resin and infiltrated overnight on a rotator. Next, 100% Epon/Araldite resin was added and rotated for 1hr at room temperature. Finally fresh resin was prepared and degassed using a vacuum chamber. The mitochondrial pellets were added to the flat molds, filled with fresh resin, and baked overnight at 60ºC. The 90 nm sections were cut using an RMC MT-7000 ultramicrotome mounted on 150 mesh copper grids and stained with uranyl acetate and lead citrate. The sections were examined using a Zeiss 902 electron microscope.

Respiration Studies:

The respiratory activity of isolated mitochondria was measured using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, England) (Brown et al. 2004; Nukala et al. 2006). 120-160 ug/mL of isolated mitochondria were suspended in a sealed, constantly stirred, and thermostatically-controlled chamber at 37ºC in respiration buffer *(*125 mM KCl, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, 0.1 % BSA, 20 mM HEPES at pH 7.2). The rate of oxygen consumption was calculated based on the slope of the response of isolated mitochondria to oxidative substrates in the order- 5 mM pyruvate and 2.5 mM malate; 150 µM ADP; 2 µM of oligomycin; 2 µM FCCP; 1µM Rotenone and 10mM succinate.

Affinity Purification by 2',5'-ADP-Sepharose Beads:

NADPH immobilized to 2',5'-ADP-Sepharose beads (50ug; Amersham Biosciences), with modifications (Lacza et al. 2003), were allowed to swell in dH_2O at 4ºC. They were centrifuged at 10,000 x g for 10 min and resuspended in binding buffer consisting of 10mM phosphate (PO^{-4}) buffer and 0.15M sodium chloride (NaCl) in 3:1 ratio. Mitochondria (1mg/ml; 50-100ug) were incubated with the beads in 1:1 ratio overnight with rocking at 4°C. The samples were

centrifuged at 1300 x g for 5min. The pelleted beads were washed 3 times with isolation buffer without EGTA and treated with sample buffer (containing SDS). The supernatants were centrifuged at 10,000 x g for 10min twice and the resulting pellet was processed for Western blots.

Proteinase-K Treatment:

Mitochondrial aliquots (100ug each at 1mg/ml concentration) were incubated with and without Proteinase-K (50-200ug/ml final; Sigma) for 30 minutes at 4°C or 25°C (Gao et al. 2004) or alternately at 37°C. Phenylmethylsulfonyl fluoride (PMSF; 1-2mM final; Roche) was added to the aliquots to stop the reaction and incubated for 10 min at 4°C or 25°C or alternately at 37°C. The samples were centrifuged at 10,000 x g for 10 min. The pellets and supernatants were collected and processed for Western blots.

Western Blotting:

Western blots for nNOS, PSD-95 and VDAC were carried out as described previously (Brown et al. 2004; Nukala et al. 2006). Briefly, the percoll-purified mitochondria were diluted to 1ug/ul in isolation buffer without EGTA and used for western blots. Sample buffer was added to the samples based on relative protein concentrations and boiled for 10min. Samples (5-20ug each) were separated by SDS-PAGE 3-8% Tris-acetate gels (NuPage), along with molecular weight markers (Multi-Marker, Invitrogen). Following SDS-PAGE, polypeptides were transferred electrophoretically onto 0.2 µm nitrocellulose membranes. Membranes were incubated at room temperature for 1-2 hour in 5% non-fat milk in 50 mM Tris-saline containing 0.05% Tween-20 at pH 7.5 (TTBS). The blots were incubated overnight in the primary antibody in TTBS at room temperature. The primary antibodies used in study included polyclonal neuronal nitric oxide synthase (nNOS) at 1:250 donated by Dr.Tim Miller; monoclonal post-synaptic density 95 (PSD-95) at 1:20,000 from BD-Transduction; monoclonal cytochrome c oxidase subunit IV (COX IV) at 1:20,000 from Molecular Probes (Eugene, OR, USA); polyclonal extracellular signal-regulated protein kinase 1/2

(Erk-1/2) at 1:1000 and polyclonal voltage-dependent anion channel (VDAC) at 1:10,000 from Affinity Bioreagents. After overnight incubation in primary antibody, the membranes were rinsed three times in TTBS and incubated in secondary antibody for 1-2 hours in either HRP-conjugated goat anti-mouse IgG (1:6000) for PSD-95, or HRP-conjugated goat anti-rabbit IgG (1:3000) for nNOS and (1:6000) for VDAC. The blots were rinsed thoroughly in TTBS and were developed using Pierce SuperSignal West Pico chemi-luminescent substrate and analyzed using Kodak Image-Station. Alternately, the nNOS and VDAC blots were incubated with fluorescent secondary anti-body (Rockland) (goat-α-rabbit, 1:10,000 in TTBS) or (1:5000) for Erk1/2 for 2hrs, with agitation, at room temperature. Blots were rinsed with TTBS, dried, scanned and quantified using a Licor scanner and Odyssey software.

NOS Activity (L-Arginine to L-Citrulline Conversion) Assay:

Briefly, increasing amounts of percoll-purified intact mitochondria were added to each reaction (total volume of 500µl) in duplicates consisting of C^{14} cocktail mix (50mM HEPES, 10mM L-Arg, 40mM $CaCl_2$, 50µM CaM, C^{14} L-Arg) in presence of NADPH and BH_4 (Bredt et al. 1990; Bredt and Snyder 1990; Miller 2002). Purified NOS is used as a positive control. The tubes are vortexed and flash centrifuged and incubated at room temperature for 15min.The reaction is stopped by adding STOP buffer (100mM HEPES; 1mM L-Cit; 10mM EDTA; pH 5.5) to each reaction. The tubes are vortexed and flash centrifuged and the contents are transferred to columns. The columns were washed with 1ml of distilled water twice. 10ul of cocktail mix is added to a BLANK vial. Approximately 8mls of ScintiSafe (Fisher SX25.5) scintillation liquid is added to each vial. The vials are read using scintillation counter and activity calculated as percent of control (no mitochondria).

Fluorescent Spectrometry to Measure Mitochondrial ROS/RNS Generation and Membrane Potential:

Mitochondrial ROS/RNS levels and membrane potential were measured using Shimadzu spectrofluorophotometer (RF-5301) as described previously (Brown et al. 2006). Mitochondria (100ug) were added to a thermostatically controlled (37ºC), constantly stirred cuvette in a total volume of 2mls of 125mM KCl buffer, containing 5mM pyruvate and 2.5mM malate, 150 µM ADP, 2 µM of oligomycin, 1mM L-Arg, 10µM H_2DCF-DA (Ex: 485nm, Em: 530nm), 150nM TMRE (Ex: 550nm, Em: 575nm) and 25U/ml HRP. Each run was performed with a baseline reading of buffer. Mitochondria, calcium and the substrates/inhibitors were added as indicated. The slope of DCF fluorescence was quantified for the respective conditions including baseline and expressed in arbitrary units.

Alternately, mitochondrial ROS production was measured using 10µM H_2DCF-DA (Ex: 485nm, Em: 530nm) and 25U/ml HRP in the Biotek Synergy HT plate reader as previously described (Brown et al. 2006). Isolated mitochondria (100ug) were added to 100 µl of 125mM KCl respiration buffer with 5 mM pyruvate and 2.5 mM malate as oxidative substrates and incubated at 37ºC. ROS production was calculated as the rate of DCF fluorescence over 15-30min of incubation, expressed in arbitrary fluorescence units.

Slot-blots for Oxidative Markers:

Samples used for respiration were centrifuged for 5min @ 10,000g. Buffer containing 0.1M PBS and protease inhibitor cocktail (Roche) was added to samples, then diluted to 200ng/ml with the protease inhibitor cocktail in 0.1M PBS. For oxidative damage determination, the OxyBlot Protein Oxidation Detection Kit (Chemicon) was used according to manufacturer's specifications. Samples were loaded at 1ug/well onto the slot blot apparatus and transferred onto 0.2µm thick nitrocellulose membrane under vacuum. The membranes were blocked for 2hrs, with agitation, in 1% Casein/PBS at room temperature. Primary antibodies were diluted in 0.1% casein/PBST and used as follows - rabbit-α-DNP (Chemicon) at 1:1000, mouse-α-3NT (Upstate) at 1:2000, rabbit-α-HNE

(Calbiochem) at 1:10,000. Blots were incubated in primary antibody overnight, with agitation, at 4ºC. Blots were rinsed with PBST and incubated Goat-α-Mouse (Jackson, for 3-NT) and Goat-α-Rabbit (Upstate; for HNE & DNP) for 2hrs, with agitation, at room temperature. Blots were rinsed with PBST, dried, scanned and quantified using a Bio-Rad scanner.

Statistical Analysis:

All results are expressed as means ± SD. For statistical evaluation, One-way analysis of variance (ANOVA) was used to test for differences involving multiple experimental groups or Student's t test was employed for data analysis involving only two groups. When required, Student-Newman-Keuls post-hoc test was used for pair-wise comparisons among multiple groups. Significance was set at $p < 0.05$ for all analyses.

Results

Characterization of Mitochondrial Preparations:

We initially characterized our mitochondrial isolation and purification methodologies by analyzing the ultra-structure of mitochondria and probing for mitochondrial markers. The electron-microscopy pictures indicate that the preparations predominantly contain mitochondria (Figure 4.1A). Mitochondria subjected to the rigorous percoll-purification maintained their structures, including intact outer and inner mitochondrial membranes as well as cristae (Figure 4.1B & 4.1C). Mitochondrial marker proteins (VDAC and COX) were probed by western blots in the various fractions obtained during the sequential purification steps (Figure 4.2). We observed an enrichment of both outer (VDAC) and inner (COX) membrane markers in the percoll purified fraction whereas they are markedly lower in the synaptosomal fraction.

Calcium and Mitochondrial Respiration:

To determine if calcium affects mitochondrial function, we measured mitochondrial oxygen consumption using a Clark-type electrode and observed a significant reduction of mitochondrial respiration (Figure 4.3). The reduction in respiratory control ratio (RCR; State III/State IV) was calcium dose-dependent (Figure 4.4). We then analyzed each of the substrate/inhibitor induced respiration states. We observed that Complex I (pyruvate/malate as substrates) mediated respiration was affected by calcium in a dose-dependent manner. We observed higher oxygen consumption upon addition of calcium accompanied by a reduction in State III respiration (in presence of ADP). However, State IV respiration (in presence of Oligomycin- Complex V inhibitor) was not affected. There was also a reduction in maximal oxygen consumption upon addition of the uncoupler FCCP. There were no changes observed in the presence of Rotenone (Complex I inhibitor) or Complex II mediated respiration (succinate as substrate) (Figure 4.5). Lastly, we observed an incubation time-dependent effect of calcium on the mitochondria in State III, State IV and State V respiration. Interestingly, we again did not observe changes in succinate-driven respiration (Figure 4.6).

Mitochondria and NOS Protein:

To determine whether a nitric oxide Synthase (NOS) isoform was present in the mitochondria, we probed for NOS using Western blots. We were able to detect NOS protein in percoll-purified intact mitochondrial fraction using an antibody directed against neuronal NOS (nNOS), while PSD-95 is present in the synaptosomal but absent in the mitochondrial fraction (Figure 4.7). We were able to detect increased intensity of NOS protein as a function of the antibody dilution (Figure 4.8A). To rule out contamination by cytosolic NOS, we incubated mitochondria with 2',5'-ADP Sepharose beads, which binds to any extra-mitochondrial NOS, and were able to detect the protein in the mitochondrial fraction (Figure 4.8B). Further, this protein band was detectable even after the addition of Proteinase-K, which digests any protein bound to the outer mitochondrial membrane, indicating that NOS has an intra-mitochondrial

localization (Figure 4.9A). Lastly, Proteinase-K was able to digest Erk1/Erk2 (cytosolic marker) completely in presence of Triton-X, a detergent used to permeabilize membranes (Figure 4.9B).

Mitochondria and NOS Activity:

Based on the data indicating the presence of NOS protein in the mitochondria and to test the involvement of nitric oxide synthase (NOS) in calcium-mediated inhibition of mitochondrial respiration, we conducted experiments to determine NOS activity in mitochondria. We were able to measure enzyme activity of NOS in percoll-purified intact mitochondria, as detected by radiolabeled L-Arg to L-Cit conversion assay and observed an increase in NOS activity dependent on the amount of mitochondrial protein (Figure 4.10).

Calcium and Mitochondrial ROS/RNS Generation:

To determine if calcium could induce ROS/RNS generation in the mitochondria, we used dihydro-dichlorofluorescin diacetate (H_2DCF-DA) to measure free radical generation. We observed a significant increase in the DCF fluorescence of mitochondria in presence of calcium (200nmol/mg) compared to those without calcium (Figure 4.11). To test if mitochondrial NOS might be involved in such ROS/RNS increases, in another experiment, we used a NOS inhibitor- N^G-Nitro-L-arginine methyl ester, hydrochloride (L-NAME) and a peroxynitrite scavenger - Tempol in presence of calcium (Figure 4.12). However, neither L-NAME nor Tempol decreased the calcium induced ROS/RNS levels.

Calcium and Mitochondrial Oxidative Damage Markers:

Lastly, to assess the total extent of mitochondrial oxidative damage with calcium, we utilized slot-blots to measure changes in markers of protein oxidation, lipid peroxidation and protein nitration. We observed no differences with calcium in levels of protein carbonyls, 4-hydroxynonenal (HNE) or in the levels of 3-nitrotyrosine (3-NT). We observed slight increases in levels of protein

carbonyls (Figure 4.13) at lower concentrations compared to higher concentrations of calcium. However, with 4-HNE (Figure 4.14) and 3-NT (Figure 4.15), no differences were observed at any concentrations of calcium used.

Figure 4.1: Ultra-structure of mitochondria is maintained after percoll-purification. Percoll-purified mitochondria were used to analyze their purity and ultra-structure using electron microscopy (EM). The top panel (A) is a low-magnification photograph of freshly isolated cortical mitochondria (Scale: 2cm = 2µm). The bottom panels (B) and (C) are high-magnification photographs of individual mitochondria isolated from the same preparation. Intact outer and inner mitochondrial membranes along with maintained cristae can be observed (Scale: 1cm = 0.1µm).

Figure 4.1: Ultra-structure of mitochondria is maintained after percoll-purification.

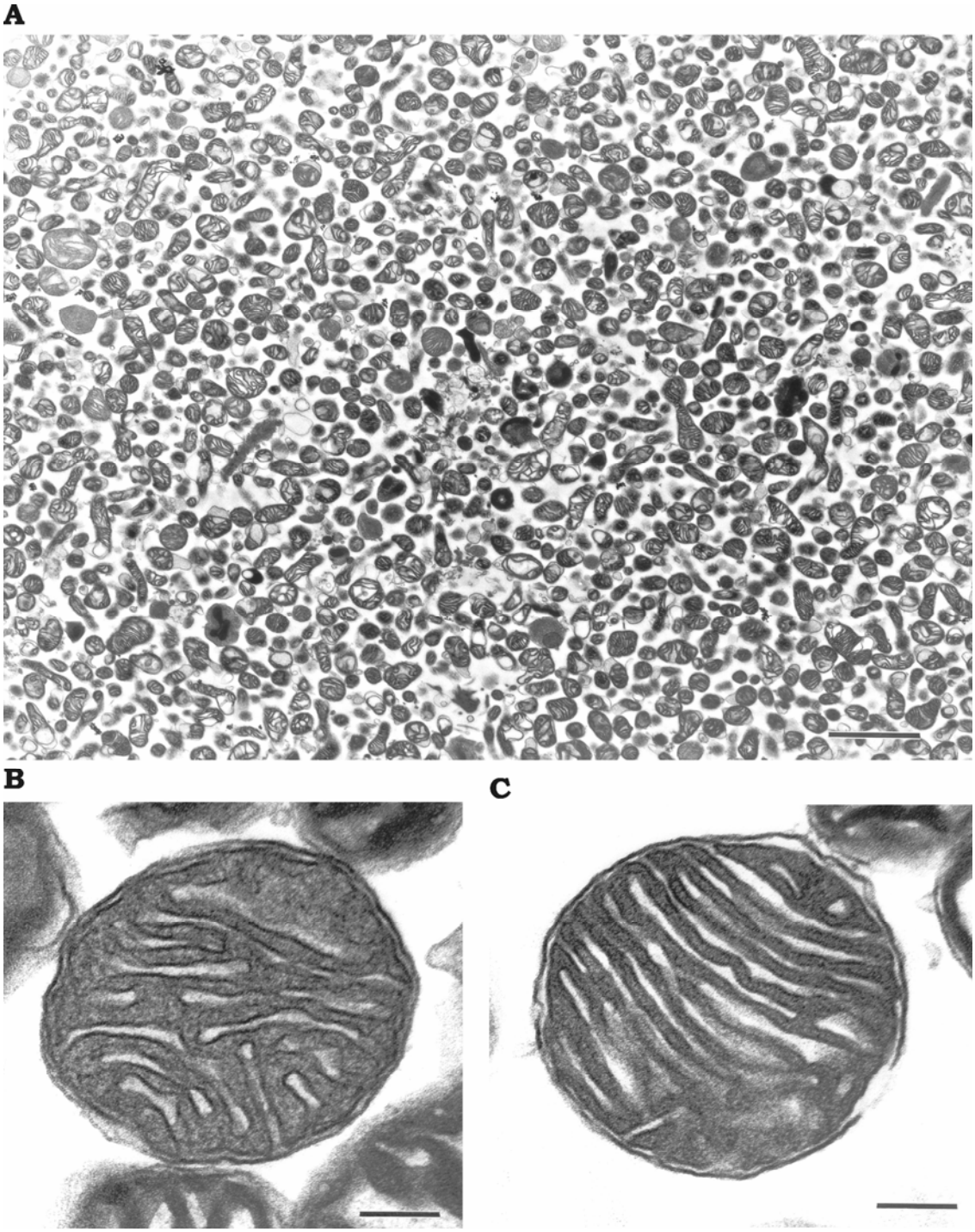


Figure 4.2: Outer and inner mitochondrial membrane marker protein levels are enriched with percoll-purification. Representative Western blots of mitochondrial markers in the various fractions collected during the isolation and purification procedure. The fractions were probed by a monoclonal antibody against cytochrome c oxidase subunit IV (COX IV or COX) at 1:20,000 and a polyclonal antibody against voltage dependent anion channel (VDAC) at 1:10,000 dilutions. The percoll-purified mitochondrial fractions demonstrate an enrichment of VDAC (outer membrane marker) and COX (inner membrane marker) protein levels compared to the brain homogenate and the crude mitochondrial fractions.

Figure 4.2: Outer and inner mitochondrial membrane marker protein levels are enriched with percoll-purification.

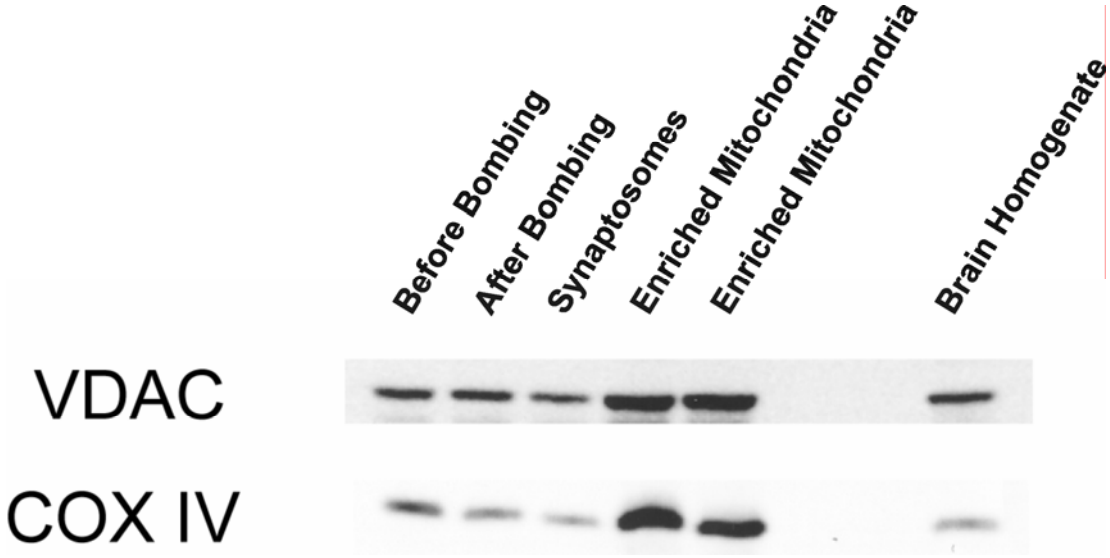


Figure 4.3: Representative traces of mitochondrial oxygen consumption with calcium. Mitochondrial respiration was carried out at 37ºC by incubating mitochondria in respiration buffer and oxygen consumption of mitochondria was measured in response to addition of various substrates and inhibitors. Baseline respiration is termed as State I containing 125mM KCl respiration buffer and mitochondria (25-30ug). State II is initiated by addition of 5mM Pyruvate and 2.5mM Malate as substrates for Complex I of the electron transport chain (ETC). Calcium was added to the mitochondria (0-1000nmol/mg) for 1min and State III is initiated by activation of ATP Synthase (Complex V) with addition of ADP (150μM). State IV occurs when ATP Synthase is inhibited by addition of Oligomycin (2μM). FCCP (2μM) induces State V by uncoupling electron transport from oxidative phosphorylation. Rotenone (1μM) is added followed by Succinate (10mM) to verify electron transport through Complex II. All respiration measurements were made in presence of 1mM L-Arg in KCl respiration buffer. Calcium inhibited respiration in a dose-dependent manner.

Figure 4.3: Representative traces of mitochondrial oxygen consumption with calcium.

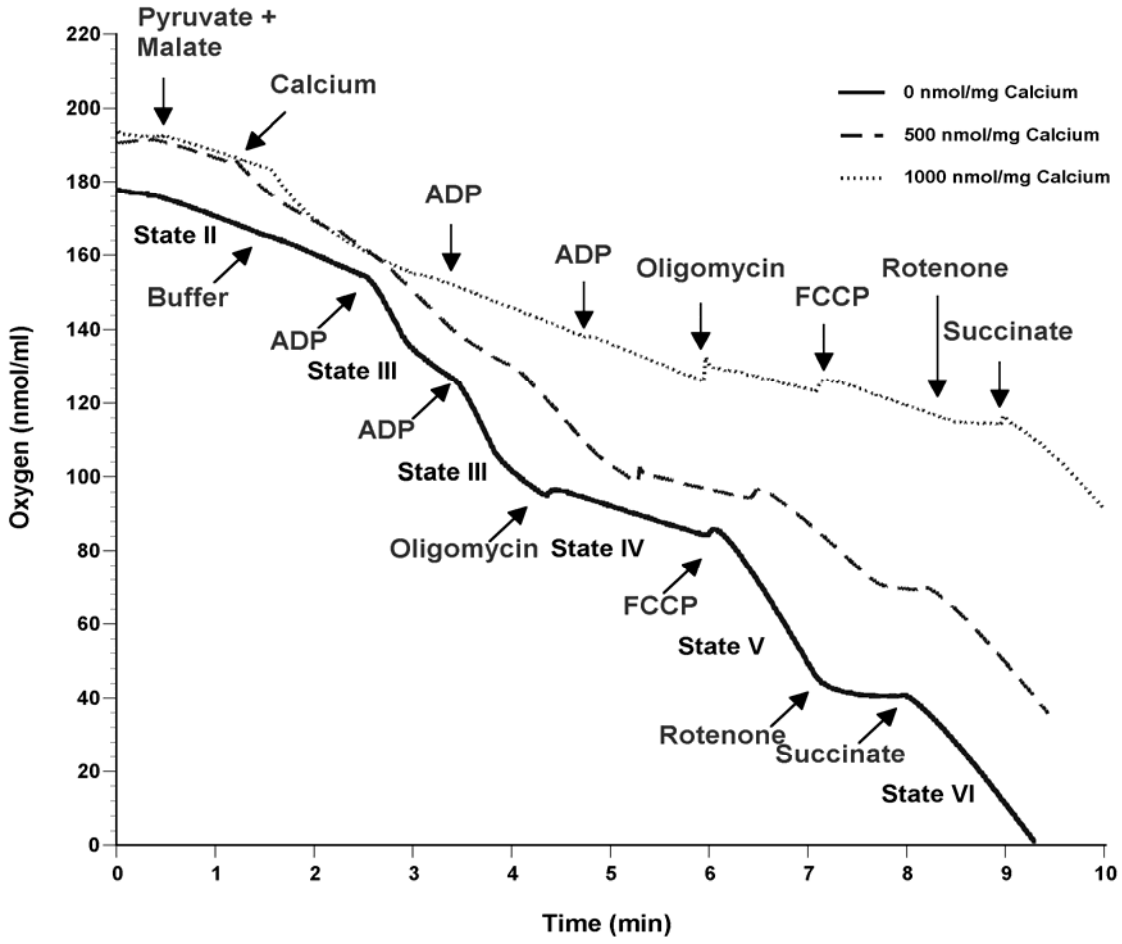


Figure 4.4: Calcium decreases mitochondrial respiratory control ratio (RCR). Increasing concentrations of calcium were used at the doses indicated and the RCR, measured as State III (presence of ADP) over State IV (presence of Oligomycin), of mitochondria in presence of calcium was significantly lowered compared to the mitochondria with no calcium. No significant differences were seen between 100 and 200nmoles/mg. Data represent group means ± SD; n = 3 per group. One-Way ANOVA; Student-Newman-Keuls post-hoc test; * $p < 0.05$ compared to 0nmol/mg Calcium; # $p < 0.05$ compared to 100nmol/mg.

Measurement	DF	F – value	P – value	Power
RCR	3	19.926	0.0005	1.000

Figure 4.4: Calcium decreases mitochondrial respiratory control ratio (RCR).

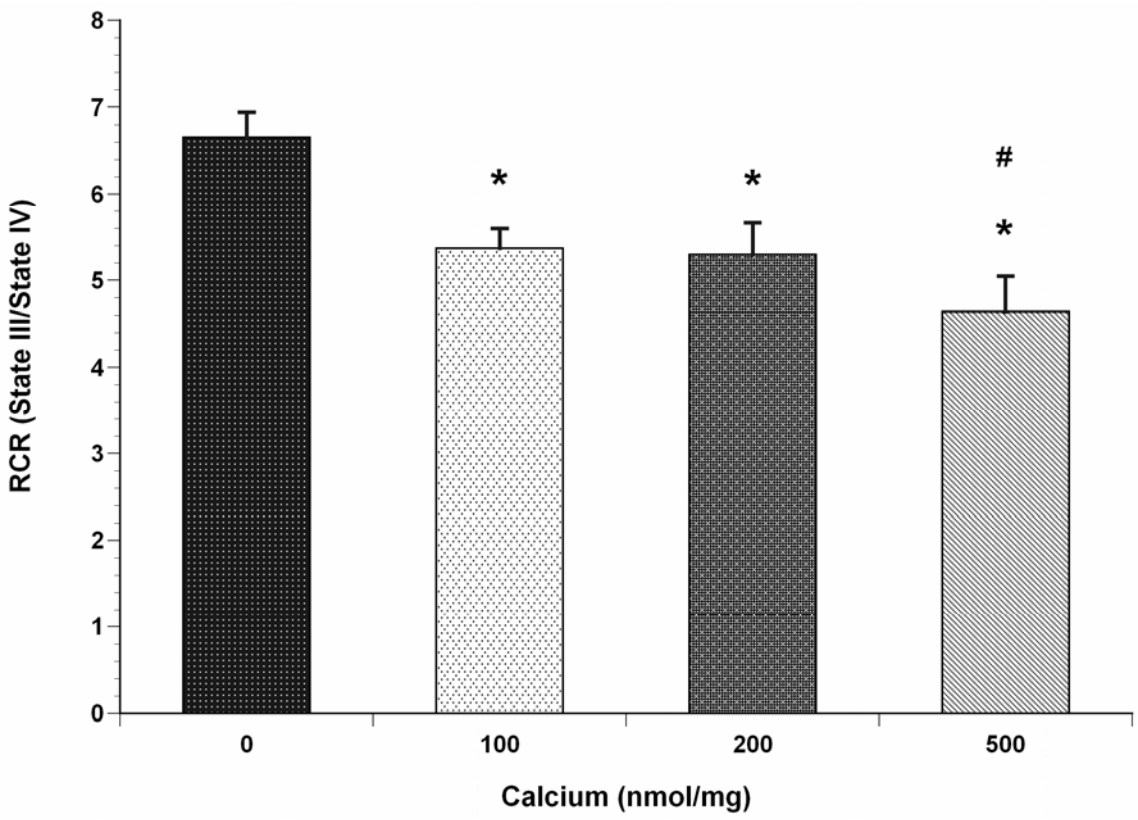


Figure 4.5: Calcium decreases mitochondrial bioenergetics in a dose-dependent manner. Increasing doses of calcium were added to the mitochondria after the addition of the complex I substrates Pyruvate & Malate. An increase in the nmol of oxygen consumed upon addition of calcium is observed, which reaches significance at 500nmol/mg. Decreases in bioenergetics was observed in State III (ADP) and State V (FCCP) as well. However, calcium did not affect the complex II (Succinate) driven respiration. Data represent group means ± SD; n = 3 per group. One-Way ANOVA; Student-Newman-Keuls post-hoc test; * $p < 0.05$ compared to 0nmol/mg Calcium; # $p < 0.05$ compared to 100nmol/mg Calcium; @ $p < 0.05$ compared to 200nmol/mg.

Measurement	DF	F – value	P – value	Power
Pyr/Mal	3	0.189	0.9009	0.072
Calcium	3	28.811	0.0001	1.000
ADP 1	3	8.318	0.0077	0.920
ADP 2	3	4.148	0.0478	0.632
Oligo	3	0.641	0.6100	0.131
FCCP	3	1.523	0.2815	0.261
Rot	3	0.647	0.6064	0.132
Succ	3	0.253	0.8568	0.080

Figure 4.5: Calcium decreases mitochondrial bioenergetics in a dose-dependent manner.

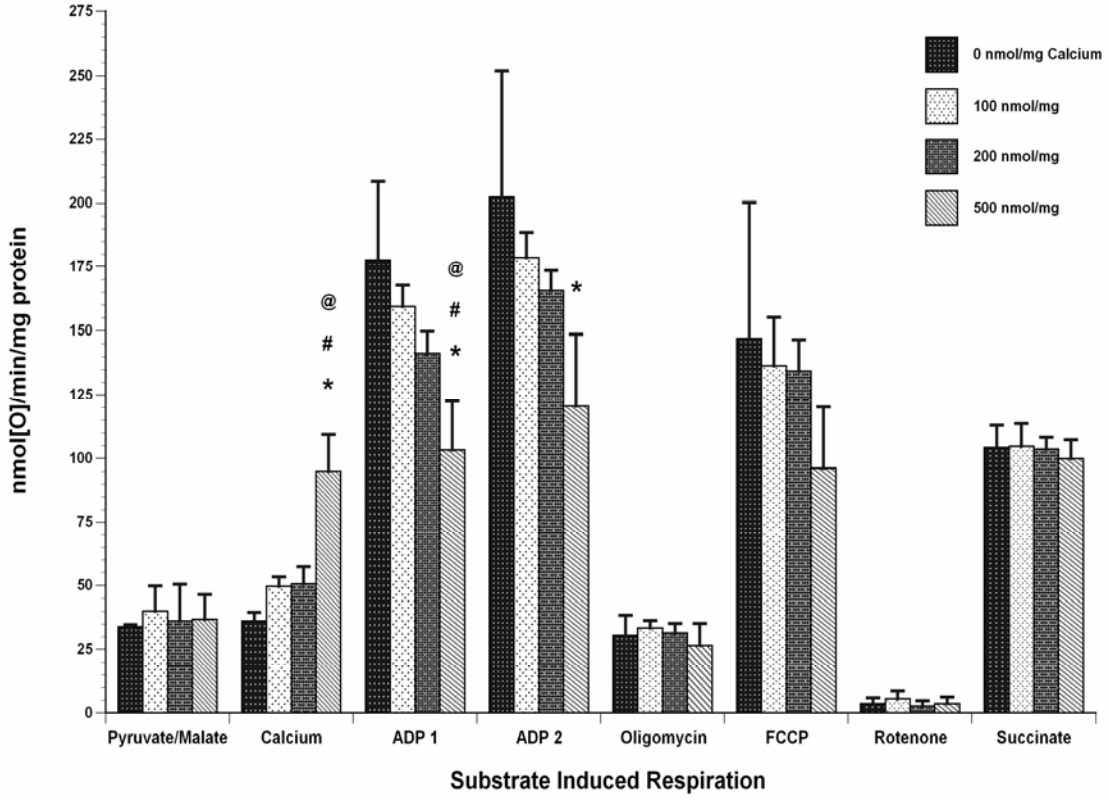


Figure 4.6: Calcium decreases mitochondrial bioenergetics in a time-dependent manner. Mitochondria were incubated with calcium after the addition of the complex I substrates (pyruvate & malate) for increasing amounts of time. An increase in the nmol of oxygen consumed upon addition of calcium was observed while State III (ADP) driven respiration was inhibited with longer incubations with calcium. When a higher concentration of calcium was added, the inhibitory effect was more pronounced. However, as seen previously in Figure 4.3, calcium did not affect the complex II (Succinate) driven oxygen consumption. n = 2 per group.

Figure 4.6: Calcium decreases mitochondrial bioenergetics in a time-dependent manner.

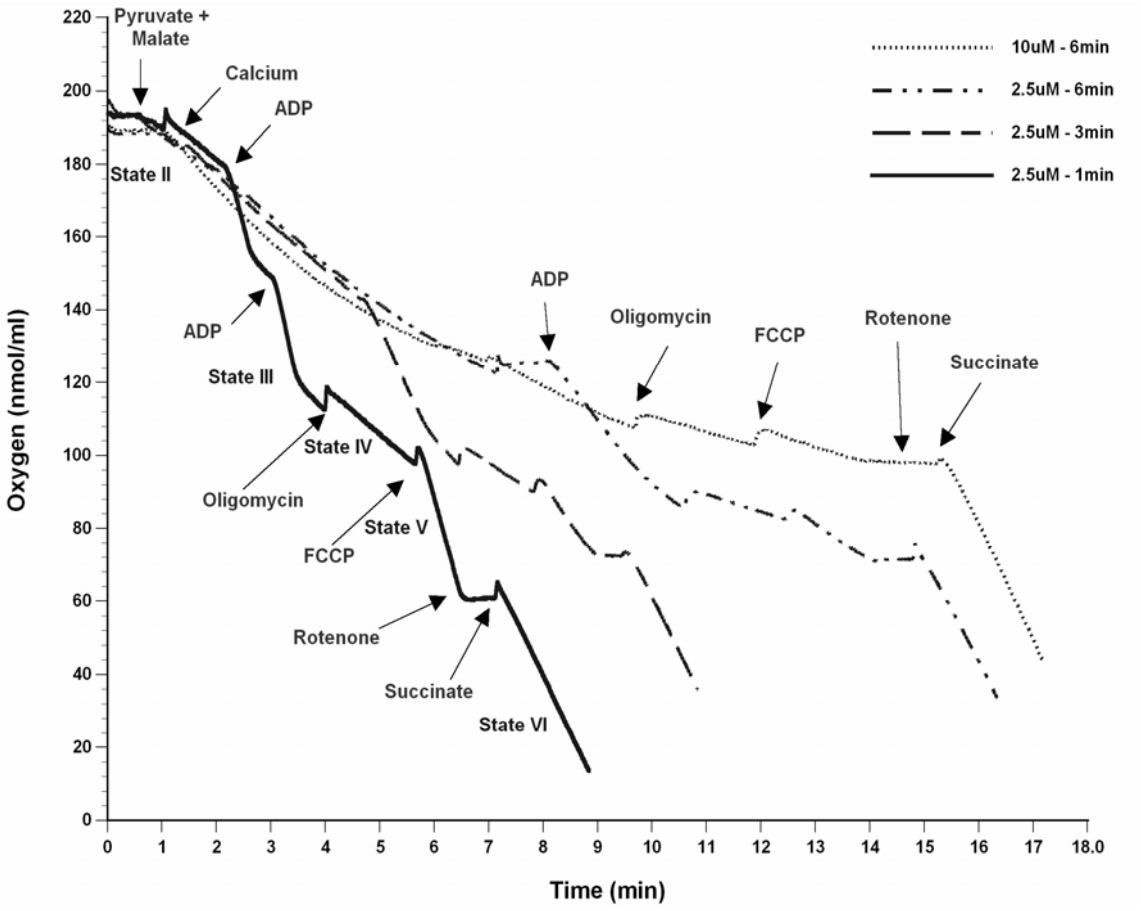


Figure 4.7: NOS protein detected in brain mitochondria. Representative Western blots indicating the purity of mitochondrial preparation. A polyclonal antibody (1:250) against neuronal NOS (nNOS) shows immunoreactivity in the percoll-purified mitochondrial fraction suggesting its presence in brain mitochondria. Post-snyaptic density 95 (PSD-95), probed by a monoclonal antibody (1:20,000), is present in the synaptosomal fraction but absent from the enriched mitochondrial fraction indicating that the mitochondrial preparation is relatively pure.

Figure 4.7: NOS protein detected in brain mitochondria.

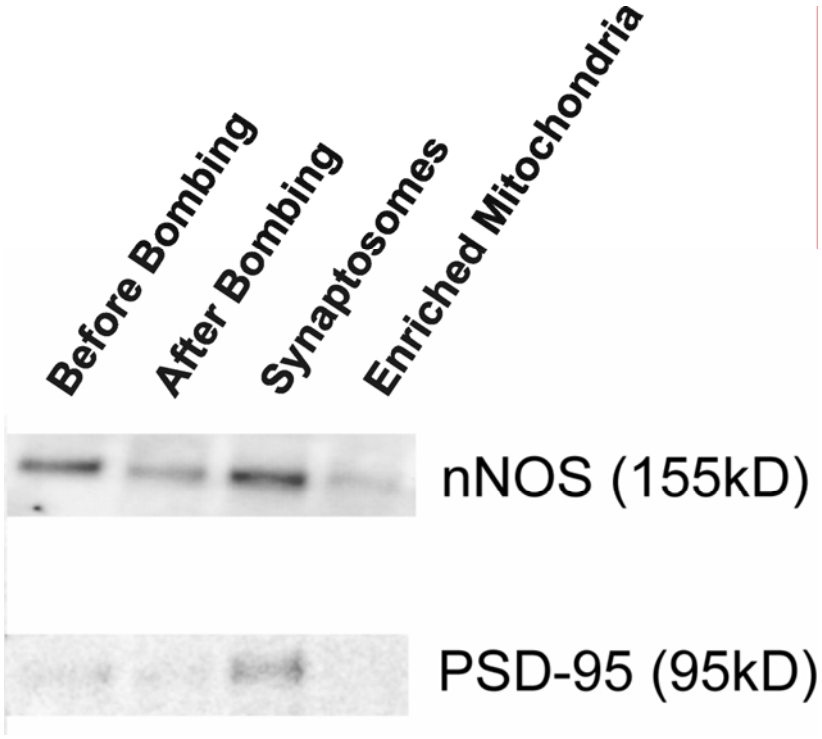


Figure 4.8: NOS protein is not an extra-mitochondrial contamination.

A) Representative Western blots showing that an intense ~155kD band was detected in percoll-purified mitochondria (5 or 10ug protein) with decreasing dilutions of a polyclonal antibody against neuronal NOS (nNOS). B) Percoll-purified mitochondria (1mg/ml; 50-100ug) were subjected to affinity purification by incubating them without (Total) or with 2',5'-ADP Sepharose beads (50ug) overnight with rocking at 4ºC to remove possible contamination by extra-mitochondrial NOS. They were then probed with antibodies against neuronal NOS (nNOS; 1:250) and voltage-dependent anion channel (VDAC; 1:10,000) using Western blots. Representative blots indicate the presence of NOS in the mitochondria treated with beads suggesting that NOS detected in the mitochondrial fraction is not an extra-mitochondrial contamination. Enrichment of (VDAC) indicates that the effect of the beads is specific to proteins requiring NADPH as a cofactor.

Figure 4.8: NOS protein is not an extra-mitochondrial contamination.

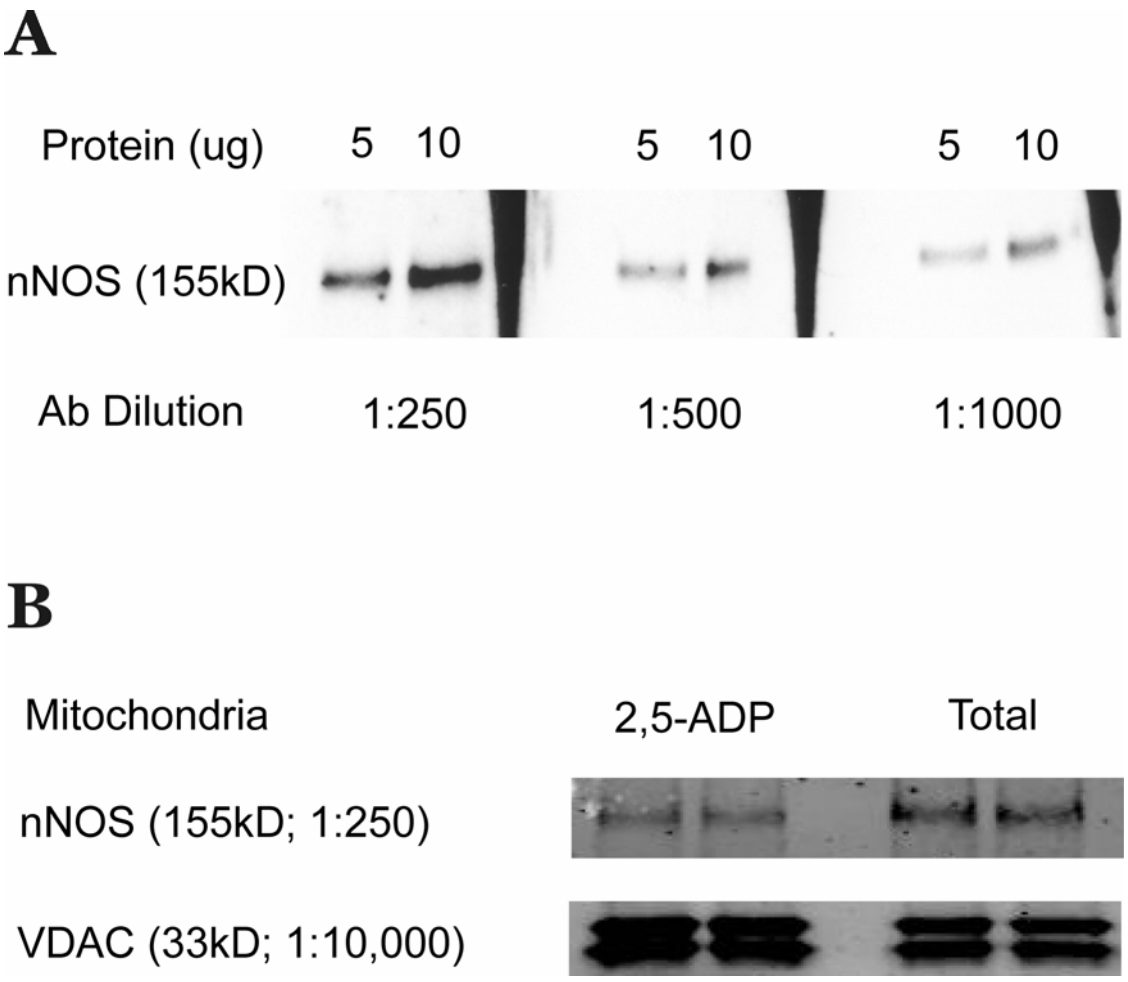


Figure 4.9: NOS protein is present in an intra-mitochondrial location.

A) Percoll-purified mitochondria (1mg/mg; 100ug) were incubated for 30min at 4ºC or 25ºC in absence or presence of a protease- Proteinase-K (50ug/ml) that can digest proteins not protected by membranes. The reaction was stopped by phenylmethylsulfonyl fluoride (PMSF; 1-2mM final) incubated for 10 min at 4°C or 25°C. The samples were centrifuged at 10,000 x g for 10 min and the pellets and supernatants were collected and processed for Western blots. Antibody against neuronal NOS (nNOS) at 1:250 shows that nNOS was retained in the mitochondrial pellet fraction upon treatment with Proteinase-K, suggesting that it is present inside the mitochondria. Antibody against voltage-dependent anion channel (VDAC) at 1:10,000 indicates that VDAC was unaffected showing that it is protected by mitochondrial membranes and therefore resistant to degradation. Finally the effect of Proteinase-K degradation was not affected by the temperature of incubation (4°C or 25°C). B) Brain homogenates were incubated with Proteinase-K and/or a detergent- Triton-X (0.1%) and the reactions were carried out for 30min at 37°C. The samples were then probed using Western blots for Erk1/Erk2 (a cytosolic marker protein) using a polyclonal antibody at 1:1000. Representative blot shows that extracellular signal-regulated protein kinase 1/2 (Erk1/Erk2) was degraded partially in presence of Proteinase-K (200ug/ml) or Triton-X (0.1%) as it is not protected by membranes and completely degraded when both Proteinase-K and Triton-X (0.1%) were added.

Figure 4.9: NOS protein is present in an intra-mitochondrial location.

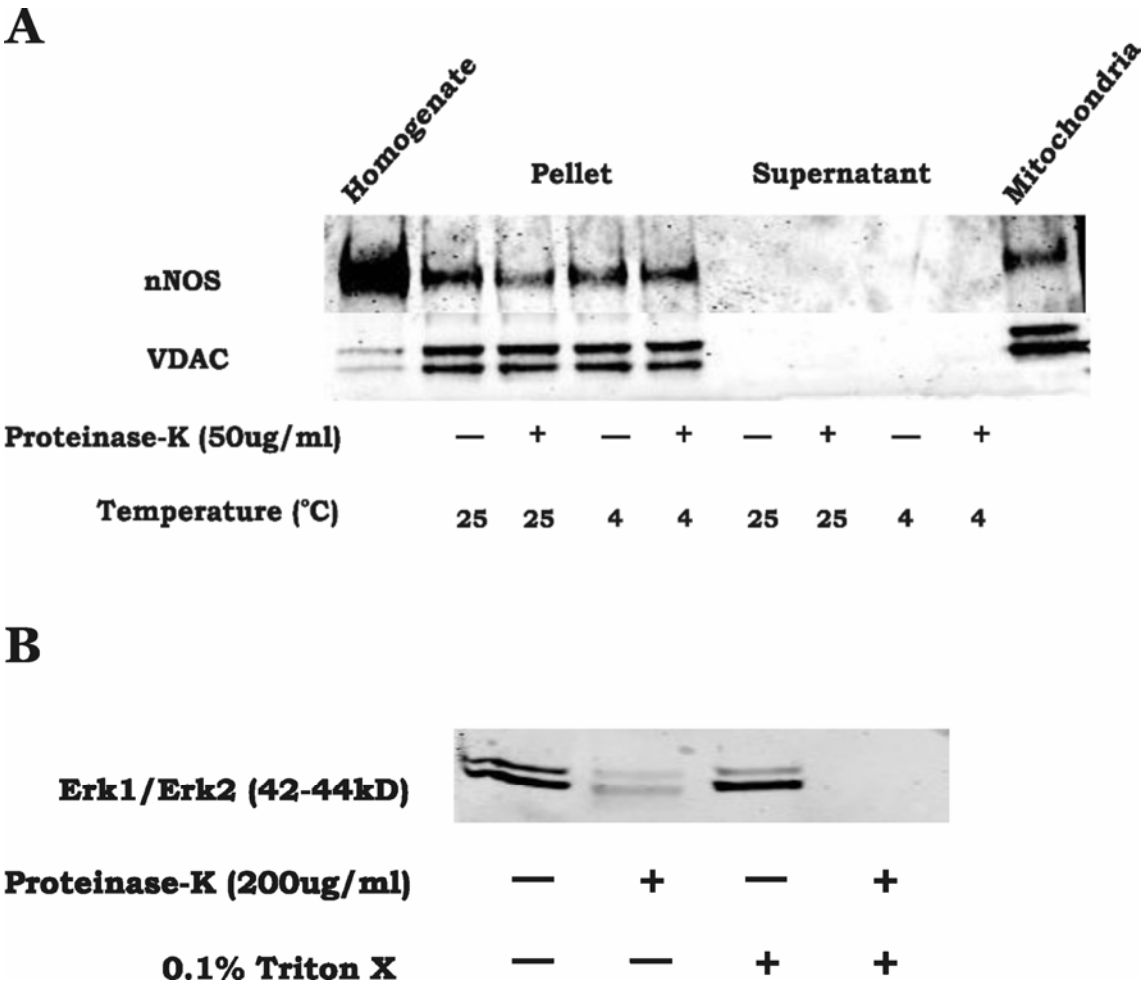


Figure 4.10: NOS activity detected in brain mitochondria. Varying amounts of percoll-purified mitochondria (0-200ug) were incubated for 15min at 25ºC in presence of NOS substrates (radio-labeled L-Arginine), cofactors (NADPH, CaM and BH_4) and activator- calcium (100μM). The reaction was stopped by adding excess of L-Citrulline and NOS activity was measured by the increase in radio-labeled Citrulline (dpm/min) produced compared to percent control (no mitochondria). Insert indicates a bar graph of NOS activity measured as nmol/min/mg of mitochondrial protein. Data represent group means ± SD; n = 4 per group; Student's t-test.

Measurement	DF	t – value	P – value
NOS Activity	6	- 2.199	0.0702

Figure 4.10: NOS activity detected in brain mitochondria.

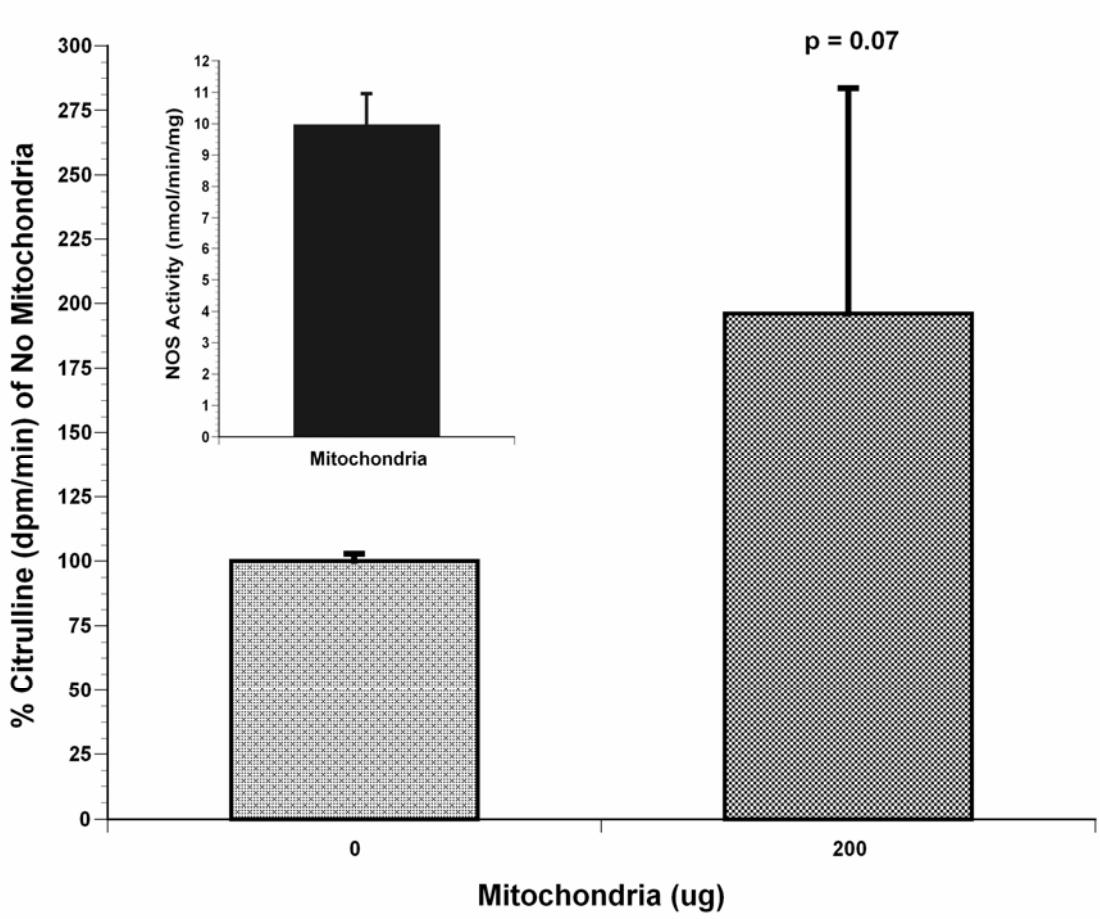


Figure 4.11: Calcium increased mitochondrial ROS/RNS levels. Mitochondria (1mg/ml; 100ug) were incubated in 125mM KCl respiration buffer for 15min at 37ºC in presence of pyruvate and malate and 10μM H_2DCF-DA (Ex: 485nm, Em: 530nm). ROS production was calculated as the rate of DCF fluorescence expressed in arbitrary fluorescence units. A significant increase in the DCF fluorescence was observed in mitochondria treated with calcium (200nmol/mg) compared to those without calcium. Data represent group means ± SD; n = 4 per group; Student's t-test; * $p < 0.05$ compared to 0nmol/mg Calcium at 15min.

Measurement	DF	t – value	P – value
DCF Fluorescence	6	- 3.112	0.0208

Figure 4.11: Calcium increased mitochondrial ROS/RNS levels.

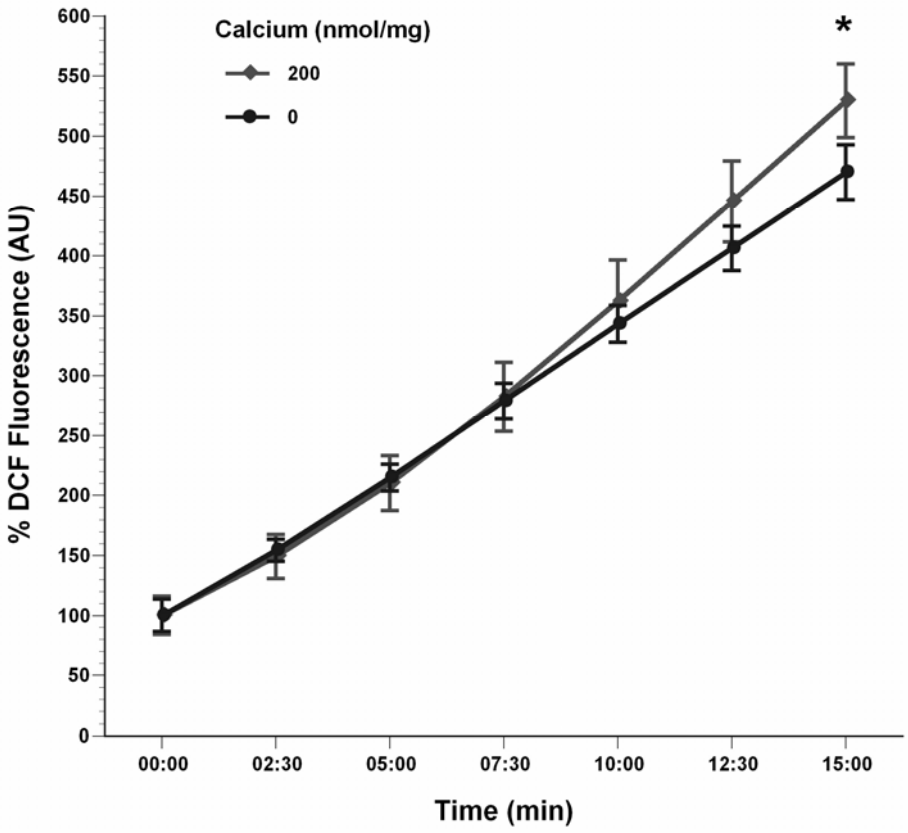


Figure 4.12: Tempol or L-NAME did not decrease calcium induced ROS/RNS levels. Mitochondria (1mg/ml; 100ug) were incubated in 125mM KCl respiration buffer for 30min at 37ºC in presence of pyruvate and malate and 10μM H_2DCF-DA (Ex: 485nm, Em: 530nm). ROS production was calculated as the percent increase in rate of DCF fluorescence compared to mitochondria with no calcium and expressed in arbitrary fluorescence units. Calcium (200nmol/mg) significantly increased ROS/RNS generation compared to mitochondria with no calcium. 100μM N^G-Nitro-L-arginine methyl ester, hydrochloride (L-NAME), a NOS inhibitor or 5μM Tempol (a peroxynitrite scavenger) did not decrease ROS/RNS levels in mitochondria treated with calcium. Data represent group means ± SD; n = 5 per group; One-Way ANOVA; Student-Newman-Keuls post-hoc test; * $p < 0.05$ compared to 0nmol/mg Calcium.

Measurement	DF	F – value	P – value	Power
DCF Fluorescence	3	8.606	0.0012	0.98

Figure 4.12: Tempol or L-NAME did not decrease calcium induced ROS/RNS levels.

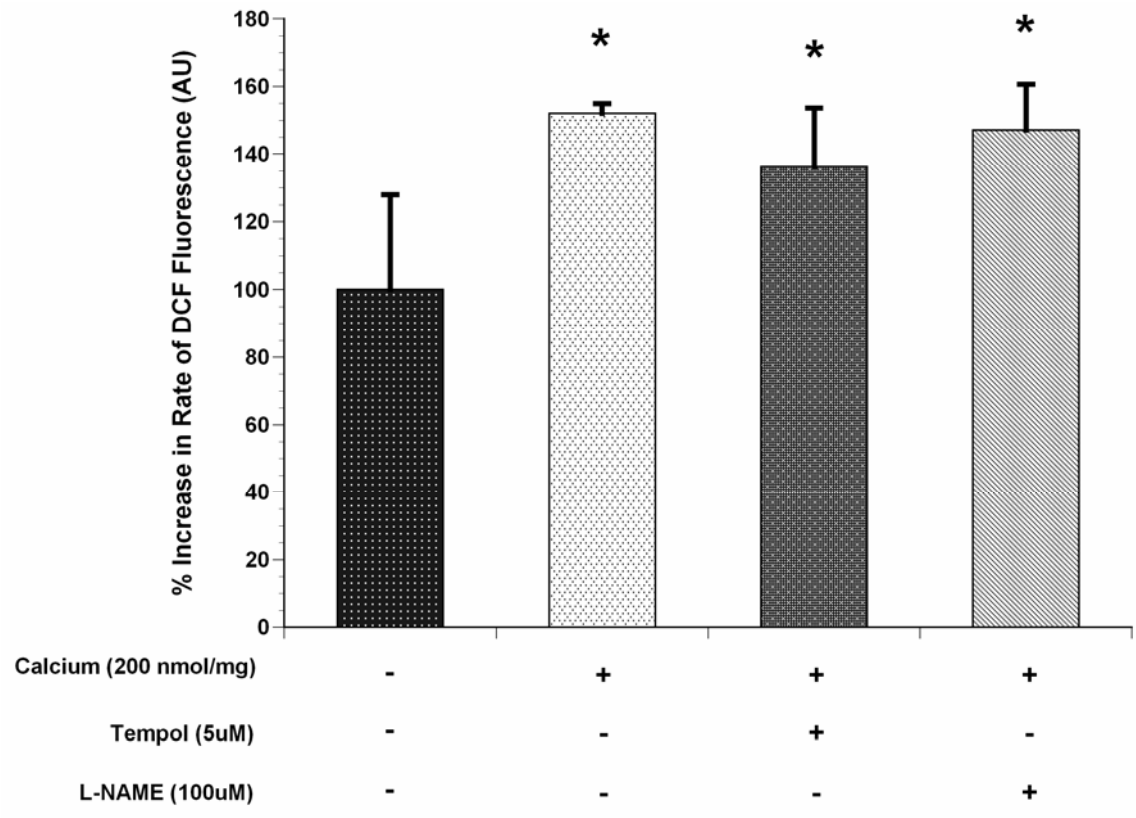


Figure 4.13: Calcium did not alter levels of protein carbonyls. Aliquots of mitochondrial samples (200ng/ml) used for respiration studies were collected and assessed for protein oxidation with an OxyBlot Protein Oxidation Detection Kit using Slot-blots. Levels of protein carbonyls were measured by a polyclonal antibody against the DNP moieties (1:1000) of oxidized proteins and expressed as percent increase compared to their levels in mitochondria without calcium. Protein carbonyls increased at lower concentrations of calcium (100nmol/mg) than at higher concentrations (200 and 500nmol/mg). However, these changes were not statistically significant compared to control (no calcium). Data represent group means ± SD; n = 3 per group; One-Way ANOVA.

Measurement	DF	F – value	P – value	Power
Protein Carbonyls	3	1.610	0.2621	0.275

Figure 4.13: Calcium did not alter levels of protein carbonyls.

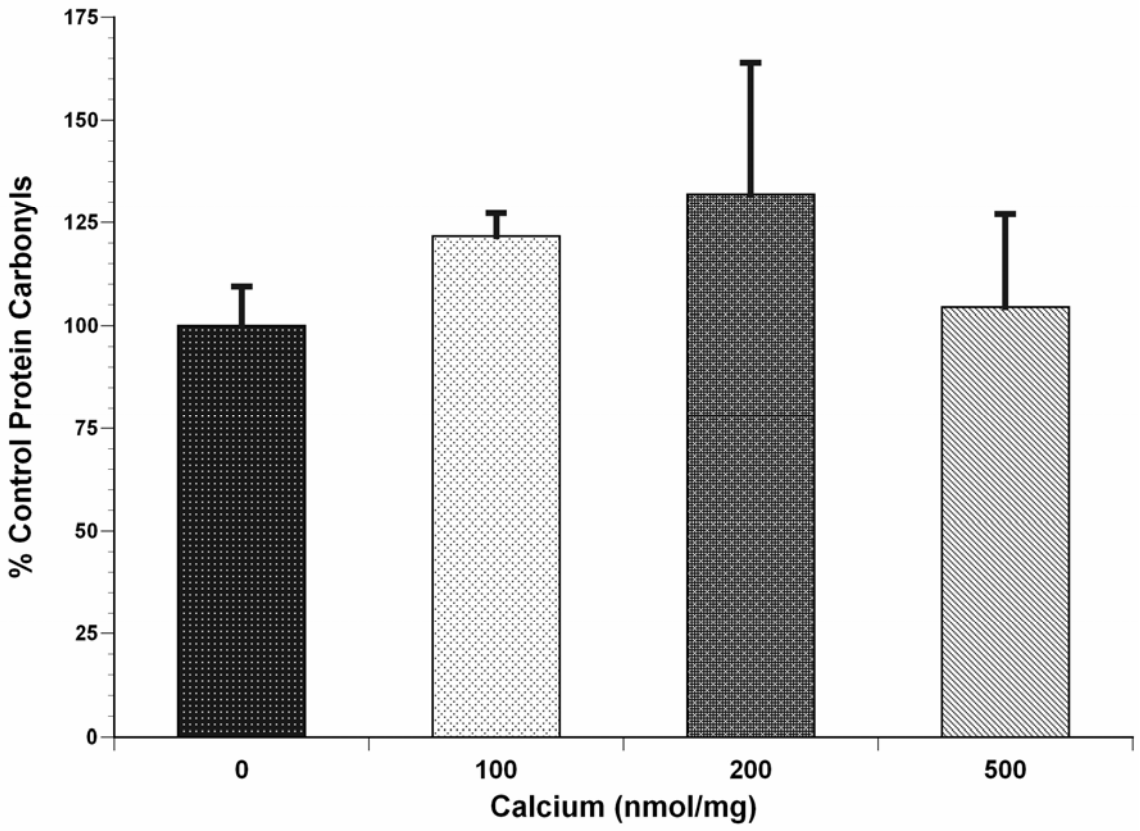


Figure 4.14: Calcium did not alter levels of 4-hydroxynonenal (4-HNE). Aliquots of mitochondrial samples (200ng/ml) used for respiration studies were collected and assessed for lipid peroxidation using Slot-blots. Levels of 4-hydroxynonenal (4-HNE) were measured by a polyclonal antibody against 4-HNE (1:10,000) and expressed as percent increase compared to their levels in mitochondria without calcium. No changes in the levels of 4-HNE were observed with any of the calcium concentrations used. Data represent group means ± SD; n = 3 per group; One-Way ANOVA.

Measurement	DF	F – value	P – value	Power
4-Hydroxynonenal	3	2.048	0.1857	0.342

Figure 4.14: Calcium did not alter levels of 4-hydroxynonenal (4-HNE).

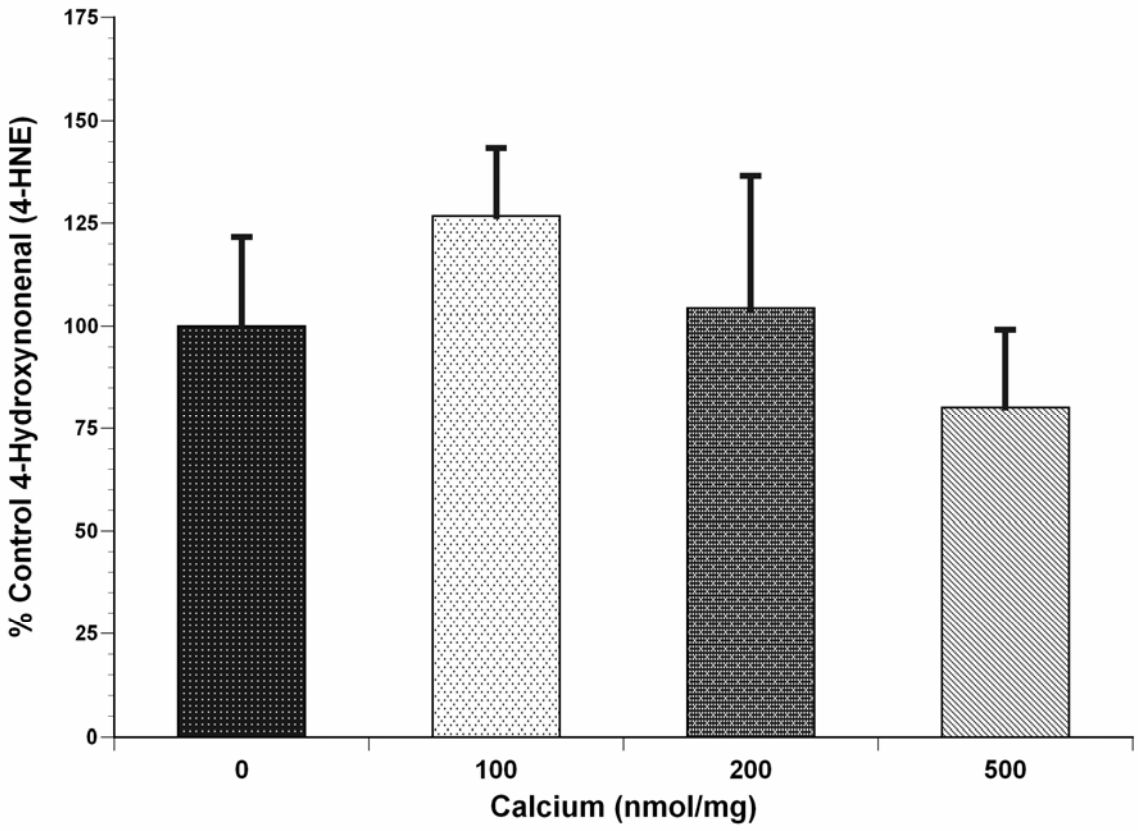
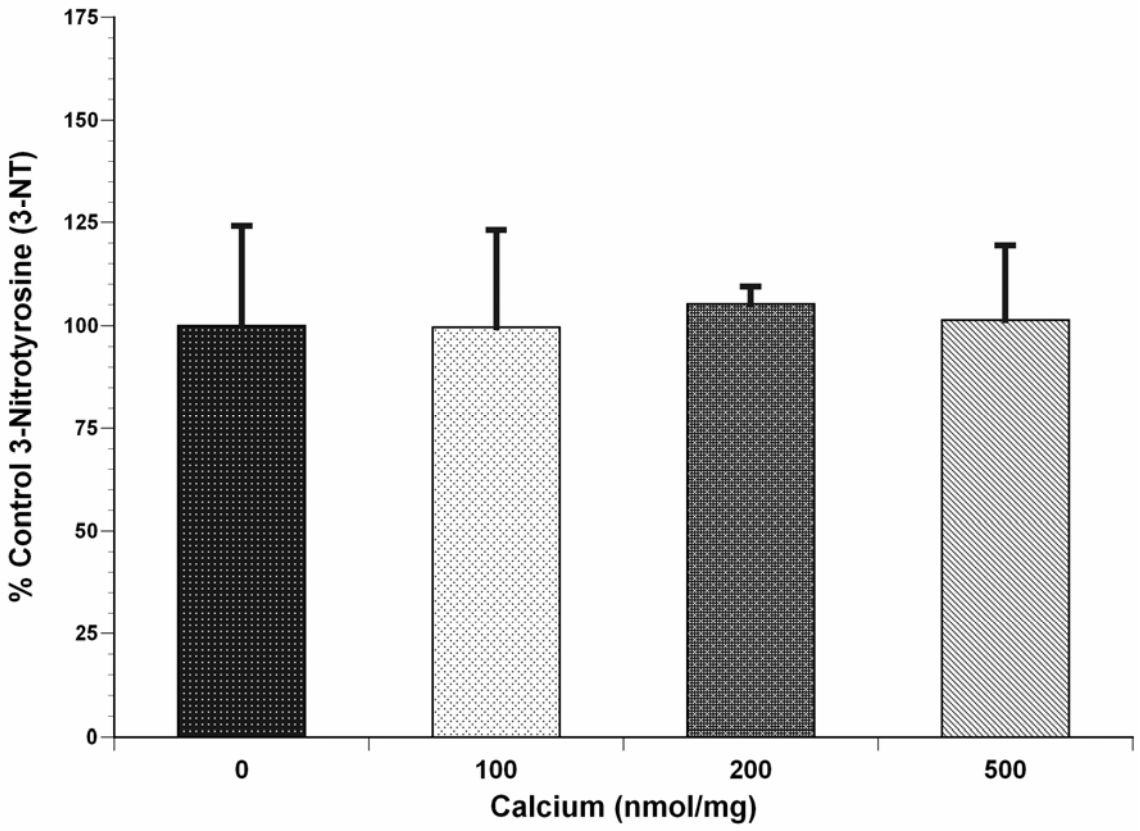


Figure 4.15: Calcium did not alter levels of 3-nitrotyrosine (3-NT). Aliquots of mitochondrial samples (200ng/ml) used for respiration studies were collected and assessed for protein nitration using Slot-blots. Levels of 3-nitrotyrosine (3-NT) were measured by a monoclonal antibody against 3-NT (1:2,000) and expressed as percent increase compared to their levels in mitochondria without calcium. Levels of 3-NT did not change at any concentrations of calcium. Data represent group means ± SD; n = 3 per group; One-Way ANOVA.

Measurement	DF	F – value	P – value	Power
3-Nitrotyrosine	3	0.051	0.9835	0.056

Figure 4.15: Calcium did not alter levels of 3-nitrotyrosine (3-NT).



Discussion

Our studies demonstrate that calcium inhibits mitochondrial respiration, and increases free radical generation. We also confirm the presence of an active NOS enzyme in the mitochondria, raising the possibility of its involvement in mitochondrial dysfunction. However, we could not improve mitochondrial bioenergetics or decrease ROS/RNS levels with NOS inhibitors or peroxynitrite scavengers. Moreover, while we did observe increases in levels of protein carbonyls (indicator of protein oxidation) and 4-Hydroxynonenal (4-HNE; indicator of lipid peroxidation) we did not observe any changes in the levels of 3-Nitrotyrosine (3-NT; indicator of protein nitration) in presence of calcium. All these results suggest that though there is an active NOS in the mitochondria, it may not be entirely responsible for the calcium induced mitochondrial dysfunction.

Mitochondria isolated from the brain are most often contaminated by synaptosomes, formed by the pinched off pre- and post-synaptic ends of neurons, that can alter and complicate the interpretation of results obtained from such mitochondria (Anderson and Sims 2000). Therefore, we employed a method to release the synaptic mitochondria with nitrogen disruption which would increase the total yield of mitochondria (Brown et al. 2004), avoiding the adverse effects of digitonin (Brustovetsky et al. 2002). We further purified the total mitochondria (synaptic and non-synaptic) by percoll discontinuous gradient centrifugation. We checked for the purity of those mitochondrial preparations by electron microscopy and found them to have mostly mitochondria with preserved structures including intact cristae that lodge the ETC necessary for oxidative phosphorylation (Figure 4.1). Moreover, mitochondrial marker proteins such as VDAC for outer and COX for inner mitochondrial membranes are enriched in the mitochondrial fraction with the sequential purification steps (Figure 4.2) while minimizing contamination. Furthermore, we used only fresh mitochondria for the studies described in this chapter, as cryopreserved mitochondria exhibit decreases in mitochondrial bioenergetics (Nukala et al. 2006).

Calcium (Ca^{2+}) is taken up by the mitochondria through a uniporter, when the cytoplasmic Ca^{2+} reaches around 1μM (Chalmers and Nicholls 2003; Nicholls 2005). Calicum is shown to regulate the citric acid cycle (TCA or Kreb's cycle) by activating pyruvate dehydrogenase (PDHC), NAD^{+}-dependent isocitrate dehydrogenase and α-ketoglutarate dehydrogenase complex (KGDHC) resulting in increases of NADH levels (Denton et al. 1988; Wan et al. 1989) as well as ATP production by activating ATP synthase (Territo et al. 2000). These reactions occur in the 0.1 to 1μM concentration range with fast kinetics on the order of milli-seconds and provide for a physiological relevance to calcium inside the mitochondria. At higher concentrations, calcium could inhibit respiration, cause mitochondria to undergo MPT and release cytochrome c ultimately leading to cell death (Gunter et al. 2004; Nicholls and Chalmers 2004; Jemmerson et al. 2005).

In our studies, we added patho-physiological doses (100 - 500nmol/mg amounting to 10 - 50μM) of calcium to isolated mitochondria and examined its effects on Complex I driven mitochondrial respiration in presence of L-Arg. We observed that calcium induced mitochondrial dysfunction and the severity was dose-dependent (Figure 4.3), similar to previous reports (Brustovetsky et al. 2003; Zhou et al. 2005). Of interest is the fact that mitochondria subjected to density gradient centrifugation are more sensitive to Ca^{2+} effects than unpurified CNS mitochondria (Brustovetsky and Dubinsky 2000). We observed significant decreases in RCR (Figure 4.4) as well as decreases in individual states, most noticeably in State III (coupled respiration in presence of ADP) and to a lesser extent in State V (maximal respiration in presence of FCCP) (Figure 4.5).

Though the oxygen consumption increased initially upon addition of calcium and returned to State II respiration rates, the response to ADP was markedly affected, and this could be due to the transient depolarizing effect seen with calcium (Figure 4.5). In one study, mitochondria exposed to a low Ca^{2+} concentration (4μM) resulted in a VDAC-mediated reversible cytochrome c release, whereas at a higher Ca^{2+} concentration (100μM), there was mitochondrial inhibition due to MPT induced irreversible cytochrome c release (Schild et al. 2001). No changes could be observed in Complex II (succinate as

the substrate) driven oxygen consumption indicating that the inhibition of mitochondrial respiration might be occurring at Complex I or upstream in the Kreb's cycle. Dubinsky's group, upon addition of calcium, has shown that cytochrome c released was accompanied by MPT and rupture of outer mitochondrial membrane. However, the decline in mitochondrial respiration was attributed to inhibition of Complex I rather than loss of cytochrome c (Brustovetsky et al. 2002), supporting our data. Further, the inhibition of mitochondrial respiration was exacerbated with longer exposures to calcium (Figure 4.6), consistent with a previous study (Territo et al. 2000). This suggests that deleterious effects of calcium are induced immediately and sustained over time. However, it is not entirely clear how calcium inhibits mitochondrial respiration and causes release of cytochrome c leaving room for the possibility of a mitochondrial NOS-mediated pathway (Lores-Arnaiz et al. 2004; Boveris et al. 2006). In fact, an involvement of NOS for the effects of calcium on mitochondrial respiration was shown in liver (Ghafourifar and Richter 1997; Giulivi 1998; Traaseth et al. 2004; Schild and Reiser 2005), heart (Jekabsone et al. 2003; Saavedra-Molina et al. 2003) and kidney (Boveris et al. 2006).

Therefore, in subsequent experiments we used two different NOS inhibitors to assess if they can reverse the inhibition of mitochondrial respiration induced by calcium (not shown). N^G-nitro-L-arginine (L-NNA) and its N^G-Nitro-L-arginine methyl ester, hydrochloride (L-NAME) are both L-Arginine analogues. L-NNA potently inhibits nNOS by binding tightly but non-covalently while L-NAME is less specific being the precursor to L-NNA (Bryk and Wolff 1999). However, we did not observe increases in State III respiration of mitochondria pre-incubated (2-5min) with various concentrations of L-NNA or L-NAME with calcium (not shown), similar to other studies in liver (Tay et al. 2004), heart (French et al. 2001) or brain (Schild et al. 2001) mitochondria. This could be due to the fact that L-Arg was present throughout the reaction and could possibly be competing with the inhibitor thereby rendering the inhibitors inefficient. We also omitted L-Arg when the inhibitors were added and it did not change the outcome, raising the possibility of the inhibitors not being sufficiently specific. Moreover, at higher

concentrations, each of the inhibitors had a toxic affect on mitochondrial bioenergetics. In another experiment, 5μM tempol, a scavenger of peroxynitrite decomposition products and acting in a catalytic manner (Carroll et al. 2000), was added to the mitochondria with calcium and tempol was unable to prevent the inhibition of mitochondrial respiration (not shown). These data suggest that the concentrations and kinetics of calcium may have an overwhelming effect on mitochondrial respiration and/or may not directly involve mtNOS. It could also be due to the differences in mitochondria from brain and other tissues from rat and other species, mitochondrial isolation and purification procedures, buffer systems and substrates and inhibitors used. Alternately, NO• or its derivatives observed might diffuse into the mitochondria and lastly, a NOS-independent pathway for generation of NO• (Zweier et al. 1999) might exist in the mitochondria.

One of the first reports for the existence of mtNOS enzyme in liver and in brain non-synaptosomal mitochondria surfaced in 1995 and indicated that it is similar to the eNOS isoform present bound to the inner mitochondrial membrane facing the matrix (Bates et al. 1995). Since then, it was suggested the isoform in liver mitochondria being similar to iNOS (Tatoyan and Giulivi 1998) while other groups have followed up showing mitochondrial isoform to be iNOS in lung mitochondria (Escames et al. 2003), eNOS (Lacza et al. 2001) and a nNOS variant (Riobo et al. 2002; Lores-Arnaiz et al. 2004) in brain mitochondria. No specific gene for mtNOS has been reported thus far and the targeting of mtNOS could involve phosphorylation or a yet undiscovered mitochondrial localization signal (Brookes 2004). Based on substrate-, cofactor- and activator-requirements and availability, it is more plausible that the NOS isoform inside the mitochondria is constitutive similar to eNOS or nNOS rather than the inducible NOS.

Therefore, to confirm the presence of NOS protein we used a polyclonal antibody against nNOS. PSD-95, a synaptosomal marker and present in the synaptosomal fraction, was absent in the mitochondrial fraction (Figure 4.7). We were able to detect NOS protein in the percoll-purified mitochondrial fraction and the intensity of the band was dependent on the antibody dilution (Figure 4.7 & 4.8A). However, the possibility of contamination of mitochondria by cytosolic

NOS still exists (Brookes 2004). To check for such contamination, we obtained percoll-purified mitochondria and subjected them to further affinity purification (Lacza et al. 2003). We treated them with NADPH (NOS cofactor) immobilized to 2',5'-ADP Sepharose beads, that will bind to any NADPH requiring enzyme bound to the outer membrane, and were still able to detect NOS (Figure 4.8B). The NOS band intensity was lower compared to untreated mitochondria while VDAC showed a much intense band, leaving a possibility for contamination. Therefore, we treated percoll-purified mitochondria with a protease (Proteinase-K), that will selectively digest proteins not protected by mitochondrial membranes (Gao et al. 2004). Indeed, we used Proteinase-K with Erk-1/Erk-2, a cytosolic marker, and it was degraded by Proteinase-K and completely digested in presence of Triton-X (Figure 4.9B). We noticed NOS protein in Proteinase-K treated mitochondria. VDAC, an outer mitochondrial membrane marker, remained intact (Figure 4.9A) indicating that the Proteinase-K did not affect mitochondrial integrity.

Giulivi's group published a contradictory report where they purified and characterized a protein from liver mitochondria and showed the isoform to be nNOSα-like with two post-translational modifications – acylation by myristic acid at the N-terminus and a phosphorylation site (Elfering et al. 2002) but do not see similar results in the heart mitochondria (French et al. 2001). Similarly, Busija's group, in a comprehensive study, question the validity of their previous claim by showing that mtNOS is not eNOS, nNOS or iNOS in liver (Lacza et al. 2003) or heart mitochondria (Csordas et al. 2007). In lieu of the divergent and confounding reports and due to non-availability of antibodies specific against mtNOS (Brookes 2004), although our data indicates a nNOS isoform present in the brain mitochondria, it does not rule out the presence of an eNOS or iNOS isoform in the mitochondria at this time.

Various groups have shown an active NOS in liver (Ghafourifar and Richter 1997; Tatoyan and Giulivi 1998; Giulivi 2003), lung (Escames et al. 2003), heart (Kanai et al. 2001), kidney (Boveris et al. 2003), muscle (Boveris et al. 2002) and brain mitochondria (Lacza et al. 2001; Riobo et al. 2002; Lores-

Arnaiz et al. 2004) while it is disputed by other groups (French et al. 2001; Lacza et al. 2003; Csordas et al. 2007). We tested if the mitochondrial nNOS detected in our brain mitochondrial preparations is physiologically relevant by measuring its activity in percoll-purified mitochondria. All the studies reporting NOS activity indicate that it shows Ca^{2+}-dependent activity and therefore we carried out all our NOS activity measurements in presence of 100μM calcium (Miller 2002). Our data shows that there is measurable nitric oxide (NO•) production (12.6 ± 0.67 nmol/min/mg) that increased with varying amounts of mitochondrial protein (Figure 4.10), suggesting the presence of a physiologically relevant NOS enzyme in brain mitochondria. The levels we detected were much higher than other reports obtained from brain mitochondria (Lacza et al. 2001; Riobo et al. 2002; Lores-Arnaiz et al. 2004). One of the reasons for this difference could be the reaction incubation times, where a longer incubation could result in a feedback inhibition of the enzyme by the NO• produced (Alderton et al. 2001). Secondly, the concentrations of Ca^{2+} and L-Arg and cofactors could alter the amount of NO generated and could explain the lack of effect of a nNOS antibody we used in another experiment. We used varying dilutions of the antibody (Riobo et al. 2002), which was used to probe for NOS protein, to inhibit the enzymatic activity but did not see any decreases in NO• production (not shown).

Mitochondria are the major sources of free radicals inside the cell formed by the leakage of electrons from various sources of the ETC (Turrens 2003) and calcium is shown to influence ROS/RNS generation in mitochondria by processes not completely understood (Camello-Almaraz et al. 2006). This is evident by the divergent reports on calcium increasing (Brustovetsky et al. 2003; Sousa et al. 2003), decreasing (Starkov et al. 2002; Gyulkhandanyan and Pennefather 2004), or causing no changes (Votyakova and Reynolds 2001; Votyakova and Reynolds 2005; Tretter and Adam-Vizi 2006) in the ROS/RNS levels of brain mitochondria. In general, from the above reports, succinate usually in presence of ETC inhibitors tends to produce higher levels of ROS/RNS with calcium compared to pyruvate/malate/glutamate as substrates.

We examined if calcium (200nmol/mg) induces or increases ROS/RNS generation within mitochondria that might be responsible for the inhibition of mitochondrial respiration. We did observe a significant increase in ROS/RNS levels with calcium (Figure 4.11) in presence of pyruvate/malate as substrates. Furthermore, the ROS/RNS levels increased over time in both groups. In another experimental protocol, we observed decreases in ROS/RNS levels with calcium accompanied by partial loss of membrane potential (Appendix 1A and 1B), supporting previous reports (Vergun and Reynolds 2005). This data is similar to the transient depolarizing effect on mitochondria seen with higher doses of calcium in our respiration studies. Our lab has previously shown that non-synaptic mitochondria are able to buffer more calcium than synaptic mitochondria before losing the membrane potential ($\Delta\psi$) and undergoing swelling but do not exhibit differences in ROS/RNS levels (Brown et al. 2006). Those mitochondria were able to sequester higher amounts of calcium compared to the doses we used in the current set of experiments. One important difference is the use of total mitochondria and the absence of ADP before the calcium challenge, especially since the presence of ADP retains mitochondrial membrane potential ($\Delta\psi$) and prevents MPT (Andreyev et al. 1998). Second, the continuous infusion method of calcium was used previously whereas bolus additions were used in all the experiments described in this chapter. Finally, we noticed that the opposite effects of calcium are in fact due to the 'effective' protein and calcium concentrations in the reaction buffers. To observe increases in ROS/RNS levels, the reactions should be carried out at higher concentrations of both the mitochondrial protein and calcium (Figure 4.11) while a reverse effect is seen with lower concentrations (Appendix 1.A & 1.B). The results from our experiments bring forth an important finding that could explain the discrepancies, about calcium effects on ROS/RNS levels, observed with the previously published literature.

It is well established that NO• at nanomolar concentrations competes with oxygen and reversibly inhibits cytochrome c oxidase (COX), acting as a physiological regulator of mitochondrial respiration (Brown 2001; Brookes et al.

2002; Moncada and Erusalimsky 2002; Babior 2004). The source of NO• was shown to be endogenous to the mitochondria (Giulivi et al. 1998) leading to decreases in ATP generation (Giulivi 1998) and increases in ROS/RNS levels (Sarkela et al. 2001), possibly due to a physical interaction between mitochondrial NOS (mtNOS) and COX (Persichini et al. 2005). However, prolonged exposure of COX to higher levels of NO• results in its irreversible inhibition caused by peroxynitrite ($ONOO^-$) (Brown and Borutaite 2002; Radi et al. 2002). Although, NO• can directly react with superoxide ($O_2^{\bullet -}$) to form $ONOO^-$, NO• can react with ubiquinol (QH_2), the reduced form of ubiquinone (Q), to yield NO^- and ubisemiquinone ($QH^\bullet$) radical. NO^- can then react with molecular oxygen (O_2) to form $ONOO^-$ (Poderoso et al. 1999). Both NO• and $ONOO^-$ are capable of diffusing across membranes and therefore into mitochondria (Radi et al. 2002). One study showed the production of $ONOO^-$ following Ca^{2+} uptake and activation of mtNOS and resulted in efflux of calcium from the mitochondria (Bringold et al. 2000). Calcium is also shown to result in release of cytochrome c (Andreyev and Fiskum 1999; Petrosillo et al. 2004) mediated through peroxynitrite (Borutaite et al. 1999; Ghafourifar et al. 1999) but independent of membrane permeability transition in brain mitochondria (Andreyev et al. 1998).

Based on those observations and our own data showing the presence of an active NOS enzyme in brain mitochondria, it raises the possibility of the involvement of $ONOO^-$ in calcium-induced free radical generation. Therefore we used L-NAME and tempol to test if they would decrease the higher ROS/RNS levels observed with calcium. We observed significant increases in ROS/RNS as seen previously in all the groups tested compared to mitochondria without calcium (Figure 4.12). Although tempol slightly decreased ROS/RNS levels compared to mitochondria treated with calcium it was not significant, similar to a previous study (Lacza et al. 2006). This is in contrast a previous report, where calcium increased ROS/RNS levels and decreased in presence of tempol (Schild and Reiser 2005). However, it was not verified if the high dose of tempol (10mM) rescued mitochondrial respiration and the effect of tempol could have been due

to its toxic effects on the mitochondria. This brings into question the role of nitric oxide (NO•) and peroxynitrite in calcium induced free radical generation in the mitochondria. Another plausible reason is that dichloro fluorescein (DCF) measures a range of ROS/RNS including H_2O_2 and $ONOO^-$ which makes it difficult to interpret the results. Moreover it is also susceptible to photo auto-oxidation (Possel et al. 1997; Ischiropoulos et al. 1999; Radi et al. 2001; Thomas et al. 2002).

Finally, we tested if the increases in ROS/RNS levels observed with calcium translates into oxidative damage in the mitochondria. We therefore measured the levels of protein carbonyls (an indicator of protein oxidation), 4-hydroxynonenal (4-HNE; indicator of lipid peroxidation). We observed differences in protein carbonyls (Figure 4.13) and 4-HNE (Figure 4.14) at lower concentrations of calcium but they were not significant. Moreover, a wide variety of nitrated proteins have been found in the mitochondria indicating involvement of peroxynitrite (Riobo et al. 2001; Elfering et al. 2004) and we therefore measured the levels of 3-nitrotyrosine (3-NT; indicator for protein nitration). However, we did not observe any changes in levels of 3-NT at any of the calcium doses tested (Figure 4.15). An absence of measurable or significant changes in oxidative damage markers does not necessarily mean the lack of RNS involvement. The methodology used measures the extent of total oxidative damage and not the individual modified proteins (Kohen and Nyska 2002; Tarpey et al. 2004). In fact, oxidative damage to certain key mitochondrial proteins including those involved in Kreb's cycle or Electron Transport Chain (ETC) has been observed. While Complexes I and II of ETC, creatine kinase are oxidatively inactivated by peroxynitrite, ATP synthase, aconitase, VDAC and Mn-SOD are inactivated by nitration of tyrosine residues through the peroxynitrite decomposition products (Radi et al. 2002). The inactivation or inhibition of these proteins might be sufficient to inhibit mitochondrial respiration (ex: ATP Synthase) or amplify the free radical generation (ex: Mn-SOD) and thereby contribute to mitochondrial dysfunction ultimately resulting in cell death.

Based on the collective evidence, although the brain mitochondria contain a nNOS-like functional enzyme it may not play a major role in calcium mediated dysfunction in brain mitochondria. This is based on the lack of effect of NOS inhibitors and/or peroxynitrite scavengers on calcium induced a) inhibition of mitochondrial respiration and b) increases in ROS/RNS levels. Moreover, no alterations were detected in levels of markers for oxidative damage. The short exposure time (less than 30min) of mitochondria to calcium might be insufficient to elicit detectable changes in ROS/RNS levels and/or oxidative damage markers.

Mitochondrial dysfunction, as measured by mitochondrial respiration, ROS/RNS levels and oxidative damage markers, is more prominent in pathologies following traumatic injuries, stroke and neurodegenerative conditions such as Alzheimer's disease (AD). This implies that calcium induced mitochondrial dysfunction may be only one of the many mechanisms responsible for the global cellular oxidative stress. One such mechanism is the cytosolic calcium induced activation of phospholipase A_2 (PLA_2) that mediates the rapid release of arachidonic acid (AA) and lysophospholipids. Some of the AA is converted to pro-inflammatory mediators such as prostaglandins, leukotrienes and thromboxanes, collectively known as eicosanoids. Arachidonic acid (AA) cascade can lead to generation of free radicals resulting in lipid peroxidation thereby altering plasma membrane as well as mitochondrial membrane composition, fluidity and permeability. The mitochondrial proteins can be oxidized from such cytosolic sources of free radicals and nitrated by cytosolic NOS mediated NO• and $ONOO^-$ generation. Lastly, calcium activated cytosolic calpains can also degrade mitochondrial proteins thereby causing mitochondrial dysfunction.

Chapter Five

Summary and Future Directions

Nature has equipped its various life forms with programmed development filled with redundancies to ensure survival and growth. There are however other stochastic events occurring in nature that modulate an organism's adaptability and thereby determine its lifespan. Free radicals are one such entity that when left unchecked can cause oxidative stress and reversible or irreversible damage to the organism. Oxidative stress at the cellular level has been implicated to have a causal role in the gradual deterioration of tissue and organ function observed with aging and age-related neurodegeneration (reviewed in Chapter One).

Typically, freshly isolated mitochondria are preferred for the study of their structure and function because such mitochondria are structurally intact as well as tightly coupled that is necessary for their respiratory function. However, in cases of a large sample size or valuable post-mortem samples or those requiring transport of samples, mitochondria isolated out of their cellular milieu undergo significant deterioration in their structure and function. This prevents conducting studies on mitochondrial bioenergetics thus limiting their usage. To overcome this obstacle, we optimized a method utilizing a well-characterized cryoprotectant such as dimethyl sulfoxide (DMSO).

Freshly isolated mitochondria were cryopreserved in the presence of DMSO and stored for the intended duration (upto 6 months). Such cryopreserved mitochondria show similar structure and function compared to freshly isolated mitochondria as observed by the ultra-structure, mitochondrial marker (COX and VDAC) levels and mitochondrial respiration. There was a partial decrease in mitochondrial bioenergetics that could be attributed to the loss of cytochrome c from cryopreserved mitochondria. They however were functionally viable compared to mitochondria frozen in the absence of DMSO. We also observed that mitochondria can be cryopreserved in their crude or purified form. This makes it feasible to considerably expand the time-frame and the range of

biochemical, molecular and metabolic studies that can performed without the constraints of mitochondrial longevity *ex vivo* (Contents of Chapter 2).

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative conditions in aged people resulting in a progressive deterioration of cognition and memory. While anatomic and metabolic deficiencies were observed with AD, the underlying mechanisms are yet unclear. Oxidative stress at the level of mitochondria and their dysfunction is one of the mechanisms gaining credibility. However, such studies are limited by the availability of suitable post-mortem brain tissues. We established a collaborative study with the Alzheimer's Disease Research Center (ADRC) Rapid Autopsy Unit of the Sander's Brown Center on Aging at University of Kentucky (Contents of Chapter 3).

We successfully employed the cryopreservation method to store mitochondria from post-mortem brains of AD and control subjects that were longitudinally assessed for neuropsychological symptoms. Synaptic and non-synaptic mitochondria were isolated from the middle-temporal (MT), frontal (F) and sensory (S) regions. The synaptic mitochondria from AD patients showed decreased respiration measured by their respiratory control ratio (RCR) and maximal complex I-driven respiration compared to control mitochondria in all the regions measured. We did not observe any changes in ROS/RNS levels. However, we observed higher oxidative damage (measured by protein carbonyls) in all the regions of AD compared to control subjects. Moreover, non-synaptic mitochondria had higher protein carbonyl content compared to synaptic mitochondria in each of the regions assessed. These data do indicate a role for mitochondrial oxidative stress and dysfunction.

Mitochondria, owing to their origin, structure and function, occupy a unique and intriguing position in the cell. They primarily provide the energy critical for metabolism, apart from calcium buffering and other vital functions. The price mitochondria pay for their semi-autonomous existence is oxidative stress. This led to a newer and significant role being ascribed to mitochondria- their involvement in cell death. Though mitochondria are established as a major source of oxidative stress inside the cell, the underlying mechanisms require

further elucidation. This dissertation attempts to offer a mechanistic explanation to the sources and the interplay of free radicals and calcium in mitochondrial dysfunction (Contents of Chapter Four).

One of the mechanisms postulated is the presence of a new isoform of nitric oxide synthase (mtNOS) that has been isolated and localized to mitochondria; though its existence and physiological role is debated. NOS, when activated by calcium, produces nitric oxide (NO•) which normally acts as a second messenger molecule in diverse cellular processes such as vasodilation and neurotransmission. NO• can also interact with ROS producing various reactive nitrogen species (RNS) such as nitrite, nitrogen dioxide radicals and peroxynitrite (ONOO-). These highly reactive radical species can damage DNA, proteins and lipids. This ultimately results in cell death via apoptosis or necrosis.

We investigated if a) calcium causes oxidative damage and mitochondrial dysfunction and b) if mitochondrial function can be rescued. We generated dose- and time-response curves for the effect of calcium on mitochondrial bioenergetics. We observed a significant reduction in mitochondrial respiratory control ratio (RCR) and rate of oxygen consumption of brain mitochondria in a dose- and time-dependent manner. This was accompanied by a transient 'depolarizing' effect on oxygen consumption with addition of calcium. This calcium effect was primarily Complex I (pyruvate and malate) driven respiration that was not observed with succinate (Complex II) driven respiration.

We then tested NOS (L-NNA & L-NAME) inhibitors in presence of calcium and measured the rescue/recovery of mitochondrial bioenergetics. We observed no recovery of State III (in presence of ADP) respiration with any of the inhibitors. At higher concentrations, each of the inhibitors had a toxic affect on mitochondrial bioenergetics. We also tested tempol, a free-radical scavenger, and observed no recovery of mitochondrial function. From these data, we conclude that calcium alters mitochondrial bioenergetics significantly and irreversibly as shown by the lack of effect of NOS inhibitors and free-radical scavengers. This could be due to lack of specificity of NOS inhibitors or more plausibly an overwhelming effect of calcium.

Furthermore, we conducted experiments to a) measure NOS activity and b) detect NOS protein in purified brain mitochondria. NOS protein could be detected in the percoll-purified mitochondria while PSD-95 was enriched in the synaptosomal fraction but visibly absent in the mitochondrial fraction. To remove possible contamination of cytosolic NOS protein in brain mitochondria, we treated purified mitochondria with cytosolic protein removal or digestion treatments. The data indicates that NOS is present inside the mitochondria though the precise intra-mitochondrial location is yet to be determined. However, it must be noted that our studies do not completely rule out contamination of mitochondrial preparations.

We measured NOS activity in isolated ultra-pure brain mitochondria and observed a mitochondrial protein-dependent increase in nitric oxide production in intact mitochondria. This suggests the presence of a NOS isoform localized to mitochondria, the exact nature of which is yet to be determined. This enzyme might be in part responsible for regulation of mitochondrial bioenergetics. However, the gene transcription, type of isoform, its transport, physiological function and regulation of protein levels and enzyme activity are all questions that need further investigation.

Experiments were then conducted to study if calcium altered ROS/RNS levels and we did observe significant increases in ROS/RNS levels over time in presence of calcium. We then tested if L-NAME or tempol would alter the ROS/RNS levels. We observed no changes in the ROS/RNS levels with either of the treatments. In a slightly different experimental paradigm, we measured ROS/RNS levels in parallel with changes in membrane potential. We did not observe any increases in ROS/RNS levels with calcium. On the contrary, ROS/RNS levels decreased significantly in presence of higher concentrations of calcium. This was accompanied by a detectable loss of membrane potential with the addition of calcium. These data suggest that the loss in membrane potential in presence of calcium might be responsible for the decrease in the observed free-radical generation. One plausible explanation could be that loss of mitochondrial membrane potential might lead to inhibition of the electron

transport chain. Studies remain to be performed if loss of membrane potential leads to membrane permeability transition (MPT) and possibly release of cytochrome c. The differences in ROS/RNS levels with calcium under various experimental paradigms could be attributed to the effective concentration of calcium and mitochondrial protein in the respiration medium. This could explain the contradictory results of various groups reporting that calcium increased or decreased or caused no change in ROS/RNS levels.

We addressed the question if changes in ROS/RNS levels with calcium led to changes in mitochondrial membrane and protein components. We tested this by measuring levels of protein carbonyls, 3-nitrotyrosine (3-NT) and 4-hydroxynonenal (4-HNE), as markers for protein oxidation, protein nitration and lipid peroxidation respectively, in presence of calcium. We observed differences in the levels of protein carbonyls with calcium but were not significant. We did not observe any changes in the levels of and 4-HNE and 3-NT, raising the question of the consequences of changes in ROS/RNS levels, the time-frame required to alter mitochondrial components and their role in mitochondrial dysfunction through oxidative damage per se.

During the course of this dissertation, one of the recurring and confounding reasons is the experimental conditions used by the various groups to determine the effects of calcium on mitochondrial respiration, presence of mtNOS and its regulation and consequences of calcium induced free radical generation. More careful studies are required to address plausible alternate mechanisms, other than acute effects of mtNOS mediated oxidative stress, underlying mitochondrial dysfunction observed with calcium.

One such mechanism is a role for nitric oxide (NO•) in mitochondrial biogenesis (Nisoli and Carruba 2006). NO• through the cyclic GMP (cGMP) pathway causes increased mitochondrial proliferation resulting in mitochondria that are functionally active. It is also shown to upregulate Hsp70, a mitochondrial chaperone involved in protein import and folding. NO• is upregulated with caloric restriction that also activates mitochondrial biogenesis implicating its role in extending lifespan. Therefore, the role of mtNOS in aging requires further

investigation. It should be kept in mind that nitric oxide (NO•) can also act as an anti-oxidant based on its local concentrations and the surrounding environment. Lastly, NO• is also involved in upregulation of uncoupling proteins (UCPs) possibly due to cross-talk between mtNOS and UCPs at the level of mitochondria.

The presence of UCPs in mitochondria adds another dimension in the regulation of mitochondrial bioenergetics (Mattiasson and Sullivan 2006). They are present in the mitochondrial inner membrane and help in translocation of protons from the inter-membrane space back into the matrix. This results in uncoupling of electron transport and oxidation from ADP phosphorylation of the ETC leading to lowering of membrane potential ($\Delta\psi$) and instead of ATP generation the energy is dissipated in the form of heat. Apart from their classic role in thermogenesis, UCPs are also shown to be involved in mitochondrial proliferation and thereby increase ATP levels. Because UCPs lower the membrane potential, they could alter mitochondrial calcium uptake and/or overload thereby preventing induction of cell death through membrane permeability transition. In addition, ketone bodies are shown to provide more NADH, substrate for Complex I driven respiration, and also lower ROS by altering the redox state of ubiquinone. Therefore, mild uncoupling through UCPs, whereby ROS generation is lowered while preserving mitochondrial function, presents a novel therapeutic opportunity in a variety of disease settings and such mechanisms are currently being investigated in the laboratory.

Appendix A

Figure A.1: Calcium decreases mitochondrial ROS/RNS levels. Mitochondria (100ug) were added to a constantly stirred cuvette in a total volume of 2mls of 125mM KCl buffer at 37ºC containing 1mM L-Arg, 10μM H_2DCF-DA (Ex: 485nm, Em: 530nm). Each run was performed with a baseline reading of buffer. Mitochondria, calcium and the substrates/inhibitors were added as indicated. The slope of DCF fluorescence was quantified for the respective conditions including baseline and expressed in arbitrary units. A significant decrease in DCF fluorescence, as a measure of free radical generation was observed in mitochondria treated with calcium compared to those without calcium. The decrease was calcium dose-dependent and was observed with additions of calcium, ADP, Oligomycin, FCCP and Rotenone but not Succinate. Data represent group means ± SD; n = 3 per group. One-Way ANOVA; Student-Newman-Keuls post-hoc test; * $p < 0.05$ compared to 0nmol/mg Calcium; @ $p < 0.05$ compared to 200nmol/mg.

Measurement	DF	F – value	P – value	Power
Mito	2	0.000	-	0.05
Pyr/Mal	2	0.574	0.5964	0.102
Calcium	2	20.738	0.0038	0.987
ADP	2	11.936	0.0125	0.889
Oligo	2	5.773	0.0502	0.594
FCCP	2	5.815	0.0496	0.597
Rot	2	3.851	0.0972	0.429
Succ	2	0.080	0.9241	0.057

Figure A.1: Calcium decreases mitochondrial ROS/RNS levels.

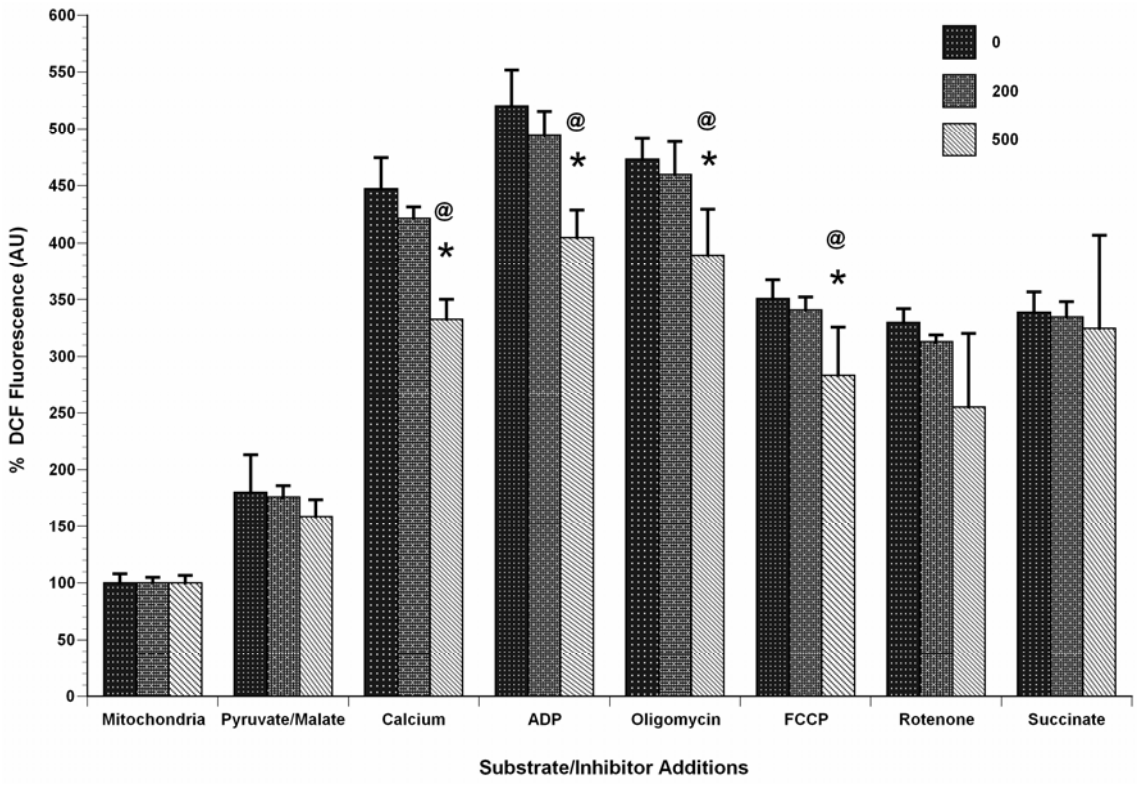
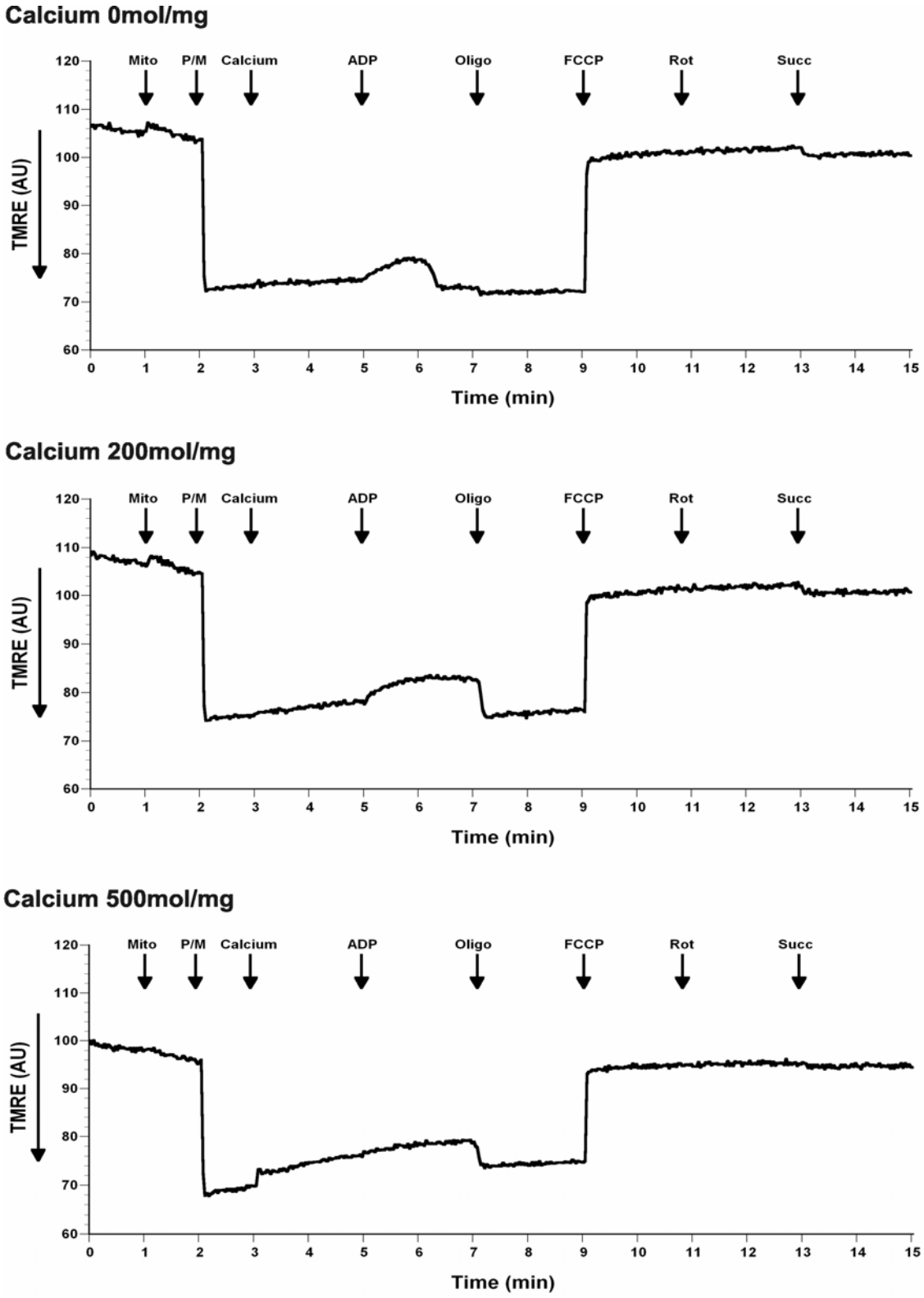


Figure A.2: Calcium decreases mitochondrial membrane potential. Mitochondria (100ug) were added to a constantly stirred cuvette in a total volume of 2mls of 125mM KCl buffer at 37ºC containing 1mM L-Arg, 150nM TMRE (Ex: 550nm, Em: 575nm). Each run was performed with a baseline reading of buffer. Mitochondria, calcium and the substrates/inhibitors were added as indicated. An increase in TMRE fluorescence, as a measure of loss of mitochondrial membrane potential ($\Delta\psi$), was observed in mitochondria treated with calcium compared to those with no calcium. This increase was calcium dose-dependent and was sustained in presence of ADP (see calcium 200-500nmol/mg). n = 3 per group.

Figure A.2: Calcium decreases mitochondrial membrane potential.



Appendix B

adenosine triphosphate (ATP), electron transport chain (ETC), calcium (Ca^{2+}), reactive oxygen species (ROS), de-oxy ribo nucleic acid (DNA), mitofusins (Mfn), dynamin-related proteins (Drp), peroxisome proliferator-activated receptor-γ coactivator (PGC), nuclear respiratory factor (NRF), outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM), inter-membrane space (IMS), mitochondrial DNA (mtDNA), ribonucleic acid (RNA), ribosomal RNA (rRNA), transfer RNA (tRNA), translocase of the outer membrane (TOM), sorting and assembly machinery (SAM), translocase of the inner membrane (TIM), nicotanamide adenine dinucleotide (NADH), flavin adenine dinucleotides ($FADH_2$), adenosine diphosphate (ADP), inorganic phosphate (Pi), NADH-ubiquinone oxidoreductase (Complex I), coenzyme Q (ubiquinone), succinate-ubiquinone oxidoreductase (Complex II), nitroproprionic acid (3-NP), Ubiquinone-cytochrome c oxidoreductase (Complex III), cytochrome c oxidase (COX or Complex IV), water (H_2O), F_0-F_1-ATPase or ATP synthase (Complex V), adenine nucleotide translocase (ANT), magnesium (Mg^{2+}), voltage-operated channels (VOC), receptor-operated channels (ROC), N-methyl-D-aspartate (NMDA), store-operated channels (SOC), receptor tyrosine kinases (TyrK), Phospholipase C (PLC), endoplasmic reticulum (ER), inositol 1,4,5-trisphosphate receptors ($InsP_3R$), ryanodine receptors (RyR), protein kinase C (PKC), protein kinase A (PKA), protein phophatases 1 and 2a (PP1 and PP2a), calmodulin (CaM), troponin C (TnC), nuclear factor-kappa B (NF-κB), nuclear factor of activated T cells (NF-AT), cyclic adenosine monophosphate (cAMP), cyclic-AMP response element binding protein (CREB), NF-kappaB inhibitory protein (IκB), calmodulin kinase II (CaMKII), sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCA), secretory-pathway Ca^{2+}-ATPases (SPCA), mitochondrial calcium uniporter (MCU), sodium (Na^+), Na^+/ Ca^{2+} exchangers (NCX), plasma membrane (PM), plasma membrane Ca^{2+}-ATPases (PMCA), proton (H^+), permeability transition pore (PTP), nitric oxide or nitrogen monoxide (NO•), endothelium-dependent relaxation factor (EDRF), superoxide ($O_2^{\bullet -}$), cyclic guanosine monophosphate

(cGMP), nitric oxide synthase (NOS), neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2), endothelial NOS (eNOS or NOS3), calmodulin (CaM), (6R)-5,6,7,8-tetrahydro-L-biopterin (BH_4), nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (Fadeel and Orrenius 2005), flavin mononucleotide (FMN), PSD-95 discs large/ZO-1 homology (PDZ), post-synaptic density protein 95 (PSD-95), heat-shock protein 90 (Hsp90), protein inhibitor of NOS (PIN), inositol 1,4,5-trisphosphate (IP_3), diacylglycerol (Arrigoni, Benzi et al. 1984), phosphatidylinositol 4,5-bisphosphate (PIP_2), protein kinase B (Akt), eNOS-interacting protein (NOSIP), eNOS traffic inducer protein (NOSTRIN), interferon-γ (IFN-γ), janus kinase (JAK), signal transducers and activators of transcription (STAT), IFN-γ regulatory factor 1 (IRF-1), NOS-associated protein 110 (NAP-110), iron-sulphur (Fe-S), heme-oxygenase 1 (HO-1), cytochrome c oxidase (COX-IV), soluble guanylyl cyclase (sGC), hypoxia-inducible factor 1 (HIF-1), iron-regulatory protein (IRP), iron-responsive elements (IREs), aryl-hydrocarbon receptor nuclear translocator (ARNT), vascular endothelial growth factor (VEGF), insulin-like growth factor II (IGF-II), cGMP-dependent protein kinase (PKG), cyclic nucleotide-gated channels (CNG), central nervous system (CNS), peripheral nervous system (PNS), long-term potentiation (LTP), lipopolysaccharide (LPS), reactive nitrogen species (RNS), nitroxyl radicals (NO^-), peroxynitrite ($ONOO^-$), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), copper,zinc-associated superoxide dismutase (Cu,Zn-SOD), free radical theory of aging (FRTA), mitochondrial theory of aging (MTA), ozone (O_3), ultra-violet radiation (UV), peroxyl radicals (ROO•), alkoxyl radicals (RO•), hypochlorous acid (HOCl), manganese-superoxide dismutase (Mn-SOD), glutathione peroxidase (GPx), nitrosoperoxo carbonate ($ONOOCO_2^-$), nitrogen dioxide ($•NO_2$), carbonate ($CO_3^{•-}$), peroxynitrous acid (ONOOH), interleukin-8 (IL-8), interleukin-1β (IL-1β), epidermal growth factor (EGF), insulin receptor kinase (IRK), mitogen-activated protein kinases (MAPK), extracellular signal-regulated protein kinase (ERK), c-Jun NH2-terminal kinase (JNK), activator protein 1 (AP-1), low-molecular-weight antioxidants (LMWA), ascorbate (Vitamin C), tocopherols (Vitamin E), glutathione (GSH), lipid radical (L•), lipid peroxyl

radical (LOO•), malonyldialdehyde (MDA), 4-hydroxynonenal (4-HNE), cysteine (Cys), methionine (Met), iron (Fe^{2+}), copper (Cu^{+}), lysine (Lys), histidine (His), 3-nitro tyrosine (3-NT), arginine (Arg), proline (Pro), threonine (Thr), tryptophan (Trp), 8-hydroxy-2-deoxyguanosine (8-OHdG), manganese (Mn^{2+}), phosphatidyl serine (PS), caspase recruitment domain (CARD), death effector domain (DED), Fas ligand (FasL), tumor necrosis factor-α (TNFα), tumor necrosis factor-α receptor 1 (TNFR1), death-inducing signaling complex (DISC), mitochondrial membrane permeability transition pore (MPTP), benzodiazepine receptor (PBR), hexokinase (HK), creatine kinase (CK), second mitocondria-derived activator of caspases/ direct IAP-binding protein with low pI (Smac/Diablo), apoptotic protease-activating factor 1 (Apaf-1), apoptosis-inducing factor (AIF), Endonuclease G (Endo G), high temperature requirement protein A2 (HtrA2/Omi), inhibitors of apoptosis proteins (IAP), voltage-dependent anion channel (VDAC), cyclophilin D (CypD), μ-calpain (calpain I), m-calpain (calpain II), Alzheimer's disease (AD), Parkinson's disease (PD), average life expectancy (ALE), maximum lifespan (MLS), mild cognitive impairment (MCI), respiratory control ratio (RCR), cryoprotective additives (CPA), dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), potassium phosphate monobasic anhydrous (KH_2PO_4), ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), hydroxyethyl piperazine-1-ethanesulfonic acid potassium salt (HEPES), magnesium chloride ($MgCl_2$), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), bicinchoninic acid (BCA), potassium chloride (KCl), enzyme-linked immunosorbent Assay (ELISA), tris-buffered saline (TBS), horseradish peroxidase (HRP), analysis of variance (ANOVA), standard deviations (SD), β-Amyloid (Aβ), positron emission tomography (PET), functional magnetic resonance imaging (fMRI), ayloid precursor protein (APP), post-mortem interval (PMI), potassium hydroxide (KOH), dihydro dichlorofluorescin diacetate (H_2DCF-DA), Alzheimer's disease research center (ADRC), frontal (F), middle-temporal (MT), sensory (S), dichloro fluorescein (DCF), arbitrary units (AU), 2,4-dinitrophenyl hydrazone (DNP), nuclear DNA (nDNA), pyruvate dehydrogenase complex (PDHC), α-ketoglutarate dehydrogenase complex (KGDHC),

mitochondrial NOS (mtNOS), calcium chloride ($CaCl_2$), L-arginine (L-Arg), L-citrulline (L-Cit), tetramethylrhodamine ethyl ester (TMRE), N^G-Nitro-L-arginine (L-NNA) and N^G-Nitro-L-arginine methyl ester, hydrochloride (L-NAME), sodium chloride (NaCl), phosphate (PO^{-4}), phenylmethylsulfonyl fluoride (PMSF), phosphate buffer saline (PBS), ubiquinol (QH_2), ubiquinone (Q), ubisemiquinone ($QH^{\bullet}$), phospholipase A_2 (PLA_2), arachidonic acid (AA)

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